DNA condensation by lipopolyamines

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DNA CONDENSATION BY LIPOPOLYAMINES

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Abstract

Small molecule lipopolyamines for DNA condensation, and potentially as vectors for non-viral gene delivery, have been designed, prepared, purified and their complexes with DNA characterised. These lipopolyamines are mimics of the naturally occurring polyamines spermine and spermidine, conjugated to hydrophobic steroid moieties. Unsymmetrical polyamine-steroid conjugates have been synthesised using regiochemically-controlled protections of symmetrical spermine or homologation of the polyamine chain by reductive alkylation. Acylation with cholesterol, ergosterol and lithocholic acid then afforded cholesteryl and ergosteryl carbamates and lithocholic acid amides of polyammonium spermidine and spermine mimics.

Polyamines and lipopolyamines have been assayed for their ability to condense natural and synthetic DNA, and compared to poly-L-lysine by means of normal absorption- and fluorescence-based spectroscopic techniques. DNA condensation was assessed using an ethidium bromide fluorescence displacement assay, and has been correlated to particle formation by light scattering techniques in the context of lipoplex formation for gene delivery.

Fluorescent derivatives of these lipopolyamines have been designed. The synthesis of fluorescent cholesteryl-polyamine carbamates required a regio- and steroselective hydroboration of these alkenes. The major product was a 6α-hydroxy, *trans*-AB steroid. By preparing Fmoc protected 5-aminopentanoic acid esters of these hydroborated cholesteryl carbamates and also polyamine-lithocholic acid amides, the incorporation of fluorescent labels was achieved via Fmoc-deprotection with fluoride salts. This strategy is potentially useful for the introduction of fluorescent labels of choice and the synthesis of Oregon Green and dansyl labelled alkylspermine-cholesteryl carbamates has been achieved by this method.

Synthetic *cis*- and *trans*-AB steroidal putrescine and spermidine mimics of the natural antimicrobial product squalamine have been shown to bind to DNA by ethidium bromide displacement. Lipid shape is one of many factors affecting the DNA binding of these ligands. The *trans*-AB steroids impart greater relative binding affinity to such models for lipopolyamine-mediated DNA condensation. These SARs are important for new lipopolyamine vector design.

Naphthalimides are highly fluorescent lipophilic moieties and two examples of naphthalimide labelled alkylspermine-lithocholic acid amides have been prepared and purified. One of these, an *n*-butyl derivative has been shown, using normal absorption spectroscopy, to condense DNA with more efficiency than a long chain octadecyl analogue. These compounds and their DNA-complexes are novel fluorescent steroidal-based vectors designed as analytical tools for lipofection.

Acknowledgements

Firstly, I thank Dr. Ian Blagbrough for his expert and enthusiastic supervision and guidance throughout this project, making Bath the right choice for my PhD.

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Finally, I dedicate this thesis to the people who have been kind enough to touch me with their closest friendship during my time in Bath - Hadi, Jonathan, Caroline and Sam.

Abbreviations

AFM	atomic force microscopy
Boc	*tert*-butyloxycarbonyl
BODIPY	boron dipyrromethene difluoride
C	cysteine
ct-DNA	calf thymus deoxyribonucleic acid
D	aspartic acid
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOGS	dioctadecylamidoglycylspermine
DOPE	dioleoylphosphatidylethanolamine
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium
E	glutamic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
FAB MS	fast atom bombardment mass spectrometry
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid

HOBt	hydroxybenzotriazole
HRMS	high resolution mass spectrometry
IR	infra red
K	lysine
mp	melting point
NLS	nuclear localisation signal
nm	nanometre
NMR	nuclear magnetic resonance
P	proline
PEI	polyethylenimine
PLL	poly-L-lysine
poly $[d(A\text{-}T)]_2$	duplex alternating adenosine-thymidine copolymer
poly $[d(G\text{-}C)]_2$	duplex alternating guanosine-cytidine copolymer
ppm	parts per million
R	arginine
RP-HPLC	reverse phase high performance liquid chromatography
TEA	triethylamine
TFA	trifluoroacetic acid
Tren	2, 2', 2''-triaminotriethylamine
UV	ultra violet
V	valine
Y	tyrosine
Z	benzyloxycarbonyl

Contents

CHAPTER 1

Lipopolyamine-Mediated Non-Viral Gene Delivery: An Introduction

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DNA drugs for gene therapy

The term "gene therapy" describes the use of corrective genes or some other polynucleic acid agent in the treatment of disease. Central to this approach is the delivery of polynucleic acids into the nucleus of target eukaryotic cells where they will hold some therapeutic value. The process of delivering a gene suitable for expression is called transfection. In gene therapy, the delivered polynucleic acid may be a correct copy of a defective gene or could be antisense oligonucleotides designed to disrupt the disease process. This method of treatment does not seek permanent manipulation of the genome and delivered polynucleic acids can be considered ultimately to be replacing the small molecule drugs used in conventional medicine. Gene therapy is considered by many to be the future of medicine yet there exists a gulf between today's technology and the realisation of its routine use in the clinic (for recent reviews, see: Miller 1992, Anderson 1998, Li and Huang 2000). As an introduction to the work described in this thesis, we begin with a concise review and consideration of the issues currently associated with attempts to deliver genes into cells for gene therapy.

This introduction starts by describing in outline the biological role of deoxyribonucleic acid (DNA), relating organisation and function to structure and the control of this structure by large and small molecules. By describing DNA in this context, we may begin to develop DNA as a drug, which can ultimately be applied in the clinic. The delivery of such DNA for gene therapy generally requires a vector for efficient cellular transfection. However, this is not always the case and "naked DNA" has been the subject of certain, recent clinical trials in gene therapy (Anderson 1998). The majority of gene therapy trials employ a viral vector (Rosenberg *et al* 1990, Blease *et al* 1995, Bellon *et al* 1997). Due to the many, major problems (for example toxicity, immunogenicity) uncovered in these trials (Rosenberg *et al* 1990), the future for gene therapy lies in non-viral gene delivery.

Cell function by DNA structure control

Generally, eukaryotic cells are 10 to 100 μm in diameter and human cells carry DNA with a total length of 2 m in the nucleus (Davis 1999). Proteins called histones mediate the packaging of this DNA and the protein-DNA complex is known as chromatin. Chromatin assembles into bundles, nucleosomes, which coil into fibres. These fibres form loops which pack close together to form a chromosome (for a review of chromatin structure, see: Demeret *et al* 2001). This arrangement renders the DNA tightly packed, yet genes remain accessible to proteins and especially enzymes which take roles in DNA repair and replication (Davis 1999).

DNA is a copolymer of nucleotide units, each monomer linked as deoxyribose phosphodiesters. Each deoxyribose sugar is linked to a nitrogenous base, one of (usually) two purines (adenine, A and guanine, G) or two pyrimidines (thymine, T and cytosine, C). Two polynucleotide chains associate as a duplex (double helix) through the H-bonding between complementary purine-pyrimidine base pairs (adenine-thymine and guanine-cytosine), which run through the centre of the helix, surrounded by the phosphate-sugar backbone. In its most stable form under physiological conditions, the duplex is right handed, with base pairs approximately perpendicular (86°) to the helix axis. This arrangement and the conformations of the sugar units render this duplex with two grooves, a major and a minor groove. This description (Dickerson *et al* 1982) characterises B-form DNA, the common solution polymorph (Fig. 3). Other forms of DNA exist and two are discussed below.

Phosphate diesters typically have pK_as of around 1.5 (Blackburn and Gait 1990); therefore the DNA sugar-phosphate backbone is polyanionic at physiological pH (7.4). DNA therefore binds small cations such as Na^+ and Mg^{2+} which stabilise the duplex. These complexes are soluble in the aqueous media of cytoplasm and *in vitro* (Hnilica 1972). Histones are highly conserved proteins and contain a high proportion of the basic amino acids lysine and arginine, the side chains of which (containing primary amine and guanidine functional

groups respectively) are cationic at physiological pH (pK_as 10.8 and 12.5 respectively, Cowen 1997). Of the five major classes, H1 histones contain 29 % lysine (Hnilica 1972, Kasinsky *et al* 2001). These facts have prompted studies of polymers of lysine and of arginine and their binding to DNA. Poly-L-lysine (PLL, Fig. 1) mimics lysine-rich H1b histone in its binding affinity for DNA (Tsuboi and Matsuo 1966). PLL has been shown to bind preferentially to DNA rich in A-T base pairing and poly-L-arginine (Fig. 1) shows preference towards poly $[d(G\text{-}C)]_2$ over poly $[d(A\text{-}T)]_2$ (Leng and Felsenfeld 1966). Electrostatic interactions between polyanionic DNA and cationic amino acid residues of histones favour tight binding in chromatin at physiological pH and ionic strength (Matsuyama and Nagata 1970).

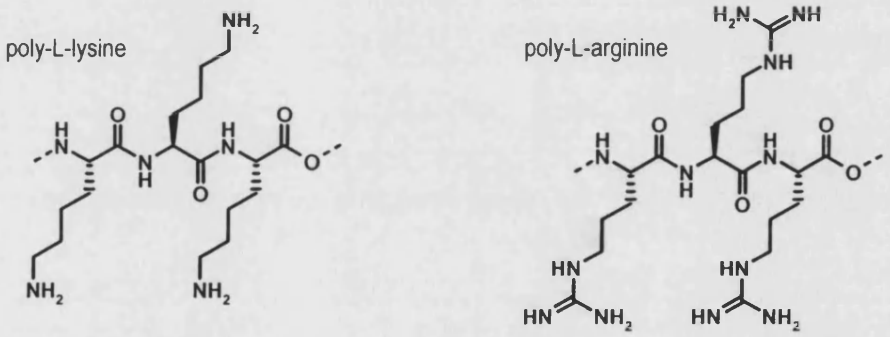


Figure 1. Poly-L-lysine and poly-L-arginine: polycationic histone mimics

Polyamines are naturally occurring small molecules (Fig. 2) and are found in high concentrations particularly in eukaryotic cells. The pK_a values of spermidine are typically 11.6, 10.8, 9.5 and 11.5, 11.0, 9.8, 8.9 for spermine (Takeda *et al* 1983). Polyamines are often essentially fully protonated under physiological conditions and these polycations also bind to DNA. Polyamines are present in chromatin and are believed to be involved in many cellular events and also to protect DNA from damage by free radicals (Ha *et al* 1998). Charge neutralisation by histones and polyamines is effective in removing electrostatic repulsions on

the DNA sugar-phosphate backbone causing local contractions of the helix and bending where this charge neutralisation occurs (Bloomfield 1996, Cohen 1998). This collapse of DNA into a compact form is called DNA condensation, and this accounts for the folded structure of DNA in cell nuclei. DNA condensation by this mechanism is said to be achieved when approximately 90 % of the negative charge is neutralised (for a review, see: Bloomfield 1997). Polyamines are also found to condense DNA in viruses (Cohen 1998).

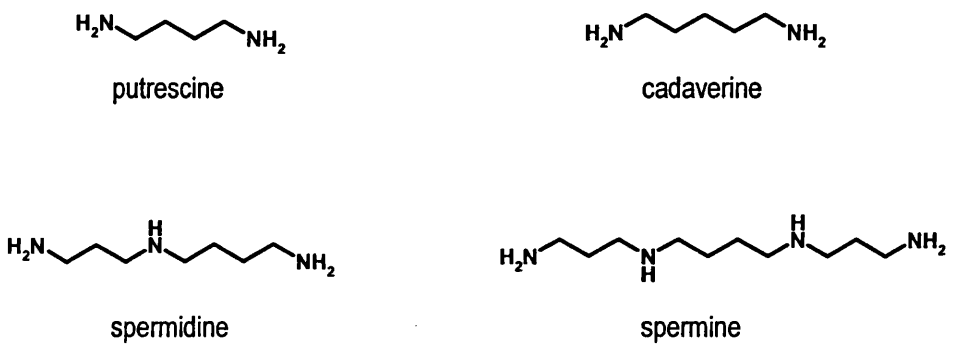
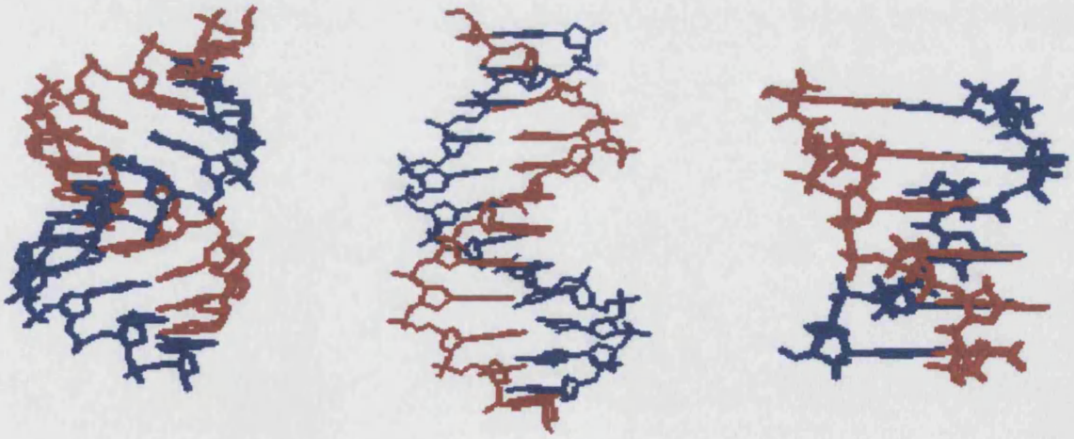


Figure 2. Some naturally occurring di- and polyamines: putrescine, cadaverine, spermidine and spermine

In contrast, bacteria (prokaryotes) lack the required amount of histone-like proteins to effect complete DNA condensation and also the polyamine spermidine (but not the diamine putrescine) makes a limited contribution (Cohen 1998). However, complete DNA condensation is observed in bacterial cells. It is thought, in this instance, that molecular crowding effects DNA condensation (Zimmerman 1993). Crowding simply implies that macromolecules (such as DNA) are compacted together by non-specific steric interactions with other macromolecules at high local concentrations in the cytoplasm. Such interactions are not sensitive to physiological salt and pH conditions as is charge neutralisation. Molecular crowding contributes mainly to the condensation of bacterial DNA into nucleoids, and is also involved in the condensation of foreign DNA introduced into bacteria by viruses (Zimmerman and Murphy 1996).

The B-form of DNA (Fig. 3) is the most commonly observed form of DNA in cells. The diamine putrescine (1,4-diaminobutane, Fig. 2), weakly stabilises the B-form, but the polyamines spermidine and spermine mediate the transition of B-DNA to the A-form (Minyat 1978, Cohen 1998). A-DNA (Fig. 3) is also a right-handed duplex, but the base pairs are tilted significantly from the helix axis and the minor groove is shallower than in B-DNA (Dickerson *et al* 1982). Short duplex oligonucleotides and RNA prefer to adopt A-form structures and, during transcription, the section of DNA being transcribed undergoes transition to the A-form to provide a closer template for the forming RNA strand (Voet and Voet 1995, Minyat 1978). It is believed that spermine and spermidine mediate this process during periods of transcription. Also, during replication, RNA primers (short pieces of RNA) must bind to sections of DNA to form RNA-DNA hybrids that have A-form structures and polyamines may be involved here. Polyamines have also been shown to mediate the transition of B-DNA to the Z-form (Dickerson *et al* 1982), a left-handed helix (Fig. 3), but the precise biological roles of Z-DNA have yet to be elucidated (Thomas *et al* 1988, Sines *et al* 2000).



A-DNA B-DNA Z-DNA

Figure 3. Molecular models of A-, B- and Z-form DNA

These structural changes to DNA are induced at low concentrations of polyamines. At higher ligand concentrations, DNA condensation begins as anionic charge neutralisation becomes more widespread on the macromolecule. When DNA is condensed with polyamines, in the absence of histones *in vitro*, toroidal-shaped particles form. Recent atomic force microscopy (AFM) studies have probed the particles formed by spermidine-mediated DNA condensation (Lin *et al* 1998). At very low concentrations of DNA (a large excess of spermidine), toroids were shown to have the same volume as a single DNA molecule (termed monomolecular, or type I toroids). At higher concentrations of DNA (but still an excess of spermidine), toroids were significantly larger and made up of smaller particles which were similar in dimension to type I. These have been termed type II, or multimolecular toroids. This evidence points to a multi-stage condensation mechanism, sensitive to changes in polyamine-DNA mixing ratios.

The binding of histones and polyamines to polynucleic acids has been described here in terms of electrostatic interactions, but polyamines are also examples of DNA groove binders. There is much evidence that polyamines reside in the grooves of DNA whilst bound, but spectroscopic and molecular modelling studies have failed to yield an exact preferred orientation (Rodger *et al* 1994 and 2000), though it is thought these molecules favour the minor groove. Many small molecule DNA-binding ligands also adopt this binding mode, for example pyrrolobenzodiazepines (Wilson *et al* 1999). Such drugs tend to have a structure that allows the molecule to follow the helix contour in the groove. DNA-binding proteins involved in gene expression processes also use the grooves in their binding regime, in particular the wider major groove of B-DNA (Creighton 1993). These proteins have a number of structural motifs that allow intimate groove binding with DNA (Adams *et al* 1992). Examples of such constructs are zinc fingers (Berg 1990), leucine zippers (Struhl 1989) and helix-turn-helix (HTH) motifs (Brennan and Matthews 1989).

Intercalation is another type of binding mode that small molecule drugs may adopt in their interactions with DNA. Planar, aromatic drugs (for example anthracyclines) may intercalate, i.e. insert between the base pairs of DNA. This type of binding will be discussed later in considering the fluorescent DNA probe ethidium bromide for the study of polyamine binding efficiency.

This section of the introduction has characterised DNA as an attractive target to a variety of molecular designs. Manipulation of this structure for determining biological function is an important consideration in the context of introducing foreign DNA for gene therapy. Knowledge of how a cell packages, organises and processes DNA is required to allow proper development of gene delivery as a technology, as this approach seeks to mimic natural cell processes (ultimately gene expression).

Gene delivery for gene therapy

Gene therapy requires the delivery of a gene or genes to the nucleus of target cells implicated in the disease, so that they may be expressed. The selection of gene or genes to be delivered is therefore decisive in determining if a condition may be treated using such a technology. The completion of the human genome project aids this selection process significantly, and genes responsible for certain diseases are now being discovered (Anderson 1998). The treatment of cystic fibrosis has become the focus of many initial gene therapy studies, such studies aiming to successfully deliver the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Such genetic material is processed as a DNA plasmid, a circular piece of DNA. The pSV-β-galactosidase plasmid (6.8×10^3 basepairs in length, Tseng *et al* 1996) is typical of a reporter gene sequence (see later in this chapter) routinely transfected by non-viral vectors.

The process of transfecting a eukaryotic cell begins with the requirement for foreign DNA to cross the plasma membrane, the first physical barrier to polynucleic acid delivery. DNA is polar and polyanionic at physiological pH and therefore free ("naked") DNA cannot easily traverse phospholipid bilayers (anionic lipids). For pieces of naked DNA (and large biopolymers generally) this represents large unfavourable molecular interactions and is one of several similar significant barriers to cross. Electroporation and microinjection of plasmids into the cytosol are techniques for this, but cannot find ready application in the genetic therapy of a disease state *in vivo*, so this introduction will focus on vectored approaches to gene delivery. Such vectors may be biological (viruses) or chemical (non-viral) and are discussed herein.

Viral vectors for gene therapy

Viruses are the natural gene delivery agents, as they rely on such processes to multiply their own numbers usually at the expense of the host cell. Recombinant viruses are commonly used to transfect cell lines and are highly efficient vehicles for gene delivery (Rosenfield *et al* 1991, Douglas and Curiel 1997). The majority of gene therapy trials to date have used viral vectors (Rosenberg *et al* 1990, Blease *et al* 1995, Bellon *et al* 1997, Zhou *et al* 1998). However, there are significant problems associated with the use of viruses in gene therapy. Expression levels of the delivered gene deteriorate within weeks of administration and patients may develop an immune response to repeat or large doses of these viruses (Walther and Stein 2000). Administration of adenoviruses has been linked to cardiotoxicity and brain damage (Pouton and Seymour 2001). Cell specific targeting is difficult to achieve with adenoviruses, a limiting factor to their systemic administration. Also, the capacity of the current viral genomes is small (around 10^3 basepairs) so the size of the DNA that can be delivered is restricted (Behr 1993).

Non-viral vectors for gene therapy

In this section we consider the cellular events that must occur for successful transfection by non-viral vectors alongside the natural cell processes that serve as barriers to this. There then follows an outline of the current approaches to transfection by the design and use of non-viral synthetic vectors. For reviews in non-viral approaches to gene therapy, see: Behr 1994, Mahato *et al* 1997, Pouton and Seymour 1998, Remy *et al* 1998.

As previously described, the first requirement of a synthetic vector is to mediate the passage of a piece of DNA across the cell membrane. Small molecules (especially lipopolyamines) and cationic polymers mimic spermine and histones (Fig. 1) in the condensation of DNA plasmids into particles. These particles are of a suitable size (typically 50-150 nm, Tang and Szoka 1997, Zelphati *et al* 1998) and charge to be taken up by endocytosis at specific points on the cell surface (clathrin coated pits, Miller 1998).

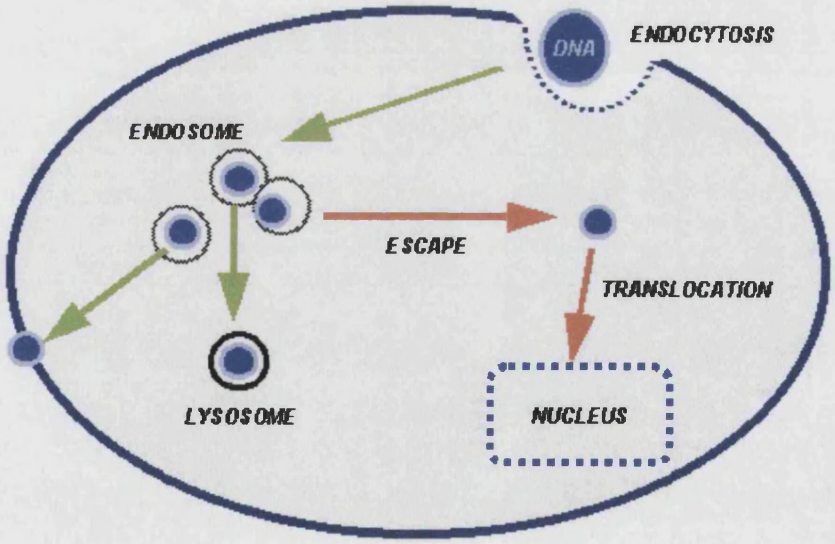


Figure 4. Schematic of default cellular processes involved in particulate uptake (green arrows) and DNA nuclear delivery pathways (red arrows)

Fig. 4 is a schematic representation of the default cellular pathways that can occur upon a particle interacting at such a site, namely endocytosis, lysosomal degradation and recycling and clearance. Also represented are the intracellular events that need to occur post-endocytosis for successful transfection, namely escape from the endosomal compartment and nuclear localisation.

DNA condensation is the first step required for efficient gene delivery, however, the natural polyamines are not suitably efficient DNA condensing agents for the delivery of DNA into cells. Therefore, polyamine conjugates with lipid moieties (lipopolyamines) and other synthetic cationic systems have been designed to achieve more efficient transfection. The following is an overview of the key compounds and concepts recently reported in the literature in this area of non-viral gene delivery.

Cationic polymers

Poly-L-lysine (PLL, Fig. 1) is a synthetic polymer, commercially available at various molecular weights and polydispersities. As introduced here, L-lysine is found extensively distributed in histones and carries a basic amine on its side-chain. PLL is therefore a polycation at physiological pH. Early work on histone-DNA interactions incorporated PLL-DNA binding studies (Leng and Felsenfeld 1966). PLL was shown to be efficient in the condensation of DNA. Since this earlier work, PLL has been used to condense DNA for gene delivery *in vitro* and *in vivo* and PLL-DNA condensates have been imaged as toroidal particles by AFM (Hansma *et al* 1998).

Starburst polyamidoamine (PAMAM) dendrimers are highly branched polymers (Kim and Zimmerman 1998). Spherical in shape, they present a surface of primary amino groups that may be protonated. Soluble in aqueous media, the cationic surface can efficiently bind to DNA and PAMAM dendrimers have been used as transfecting agents (Bielinska *et al* 1996,

Kukowska-Latallo *et al* 1996, DeLong *et al* 1997). However, efficient transfection activity has been linked with high cytotoxicity in PAMAMs and PLL (Hill *et al* 1999).

Polyethylenimine (PEI, Fig. 5) is a commercially available branched or linear polymer of aziridine. Behr and co-workers have investigated the potential of PEI as a transfecting agent (Boussif *et al* 1995). They have shown PEI to be a highly efficient vector for the delivery of polynucleotides (Pollard *et al* 1998, Godbey *et al* 1999a and 1999b and 1999c).

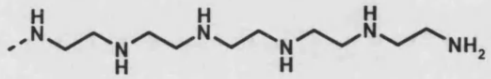


Figure 5. Linear polyethylenimine: a cationic polymer

Cationic lipids I. Long chain aliphatic lipids

The cationic lipids are a class of compounds possessing a cationic headgroup covalently bound to a lipid moiety. The cationic nature of these compounds facilitates DNA condensation by charge neutralisation and the resulting particles are cationic lipoplexes. These lipoplexes can bind to cell membranes and are taken up by endocytosis (for a review see: Miller 1998).

The first such compound for gene delivery was the unnatural diether-linked monocationic unsaturated lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA, Fig. 6) in 1987 (Felgner *et al*). Two years later the first commercially available formulation for transfection, Lipofectin, was marketed. Lipofectin is a 1:1 mixture of DOTMA and the naturally occurring zwitterionic lipid dioleoylphosphatidylethanolamine (DOPE, Fig 6. Felgner and Ringold 1989). DOTAP is the diester analogue of DOTMA and a number of related compounds (e.g. Fig. 6) and formulations have recently been developed (Ren and Liu 1999a and 1999b).

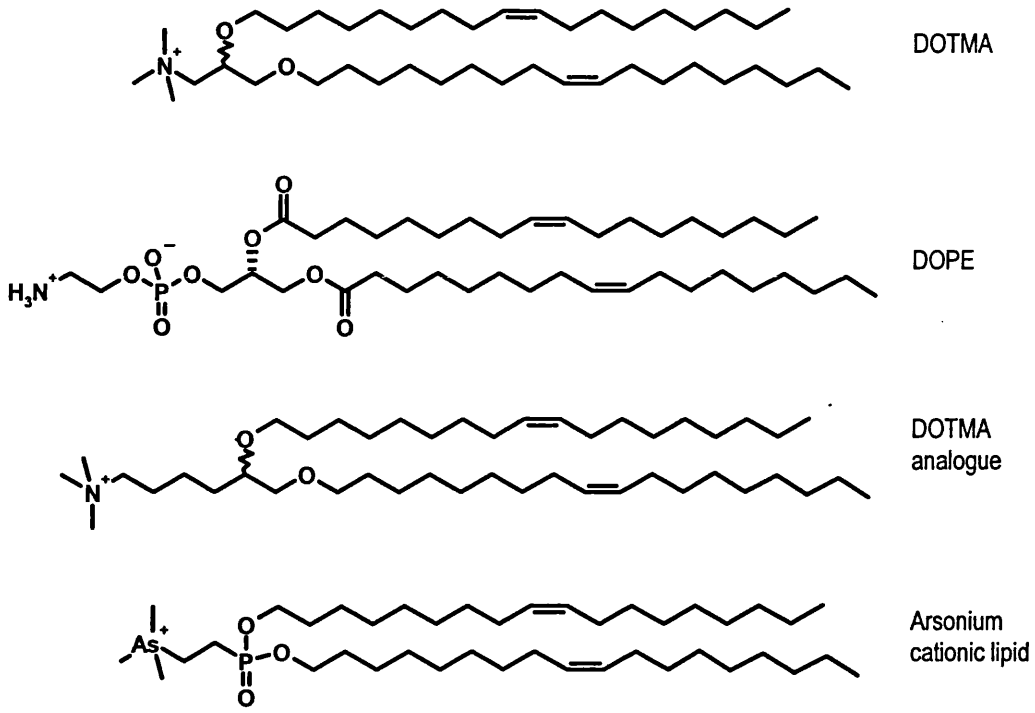


Figure 6. DOTMA and DOPE (the components of the Lipofectin reagent), a DOTMA analogue and an arsonium based cationic phosphonolipid

One of these DOTMA analogues is a cationic phosphonolipid with a quaternary arsonium moiety replacing the quaternary ammonium functional group (Guénin *et al* 2000). Arsenic occupies a greater covalent volume than fellow Group 15 element nitrogen, affording arsonium cations different ionic interactions than their ammonium counterparts, and also, potentially modified DNA binding characteristics (Guénin *et al* 2000).

Other workers have designed cationic lipids with two saturated C-18 chains. Dioctadecylamidoglycylspermine (DOGS, Transfectam, Fig. 7) is a polyamine conjugate which retains the methylene spacing of the natural DNA condensing polyamine, spermine (Behr *et al* 1989). This was the first such lipopolyamine and is an efficient gene delivery vector *in vitro* (Remy *et al* 1998). Transfectam is a commercially available reagent for transfection of cell lines. The efficiency of this system relies on an excess of Transfectam with respect to lipopolyamine-DNA charge ratio, giving cationic lipoplexes. However, this has been shown to be less effective in Transfectam mediated transfection *in vivo* (Remy *et al* 1994).

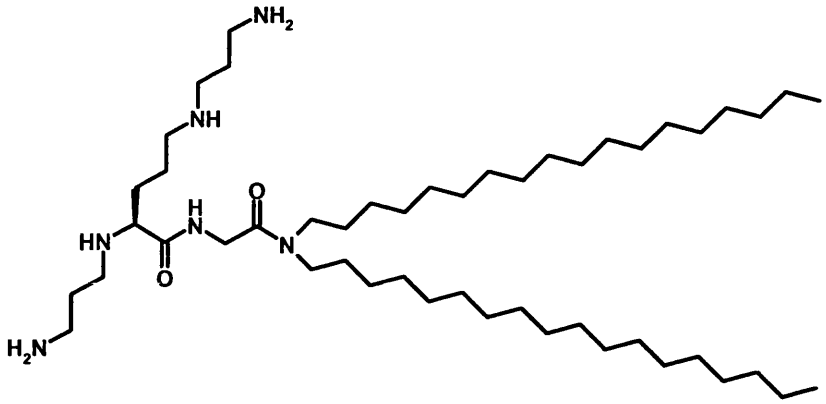
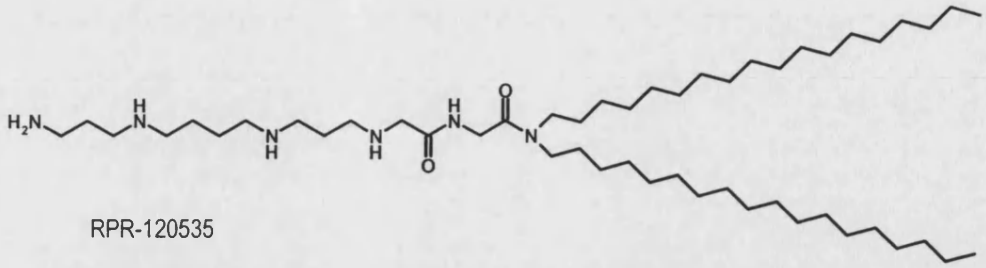


Figure 7. Transfectam (DOGS), a commercially available lipopolyamine

Byk and co-workers at Rhone-Poulenc Rorer have synthesised lipopolyamines with two C-18 lipid chains (Fig. 8), based on the established DOGS molecule (Byk *et al* 1998). Using high dilution and solid phase methodologies (Byk *et al* 1997), this research group reported a rapid preparation of a library of unsymmetrical monofunctionalised polyamines. Byk and co-workers have shown higher *in vitro* gene delivery with linear, branched and "T-shaped" polyamine headgroups compared to globular geometries and attributes this to decreased pK_a values in the globular polyamines. They (Byk *et al* 1998) also identify SARs in the linkers between the polyamine and lipid functions in these molecules. RPR-120535 is a linear polyamine linked covalently to two C-18 chains by glycine and has been shown to be the best compound in this series in the transfection of HeLa human carcinoma and NIH3T3 mouse fibroblasts with luciferase plasmid. This compound also displays significant *in vivo* transfection activity on the Lewis lung carcinoma model (Byk *et al* 1998).



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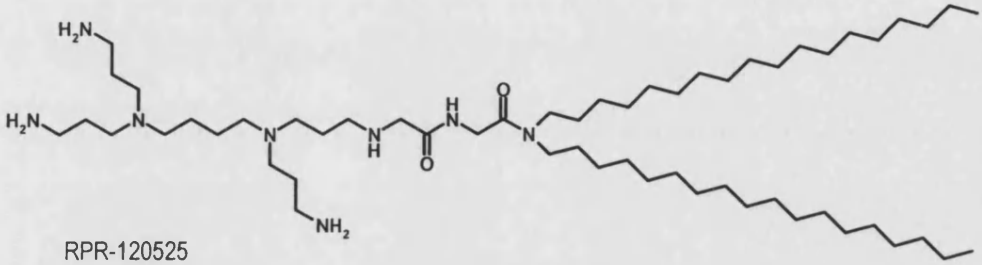

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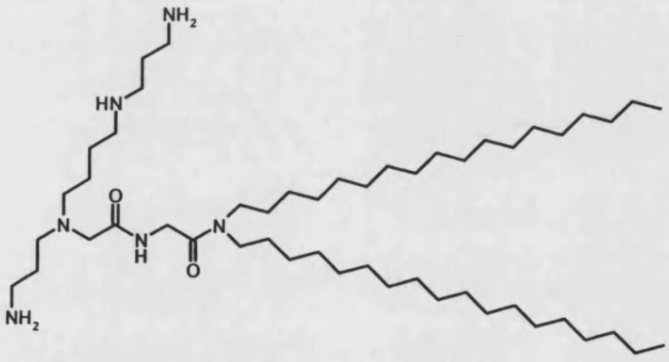

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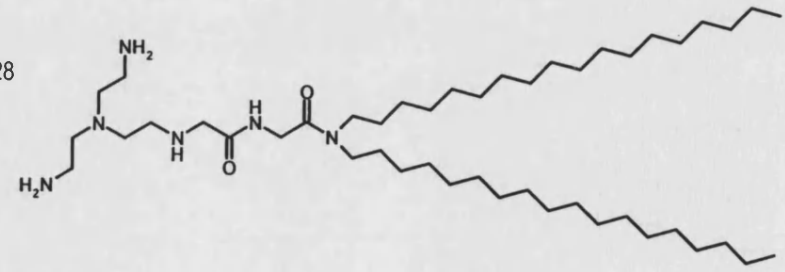
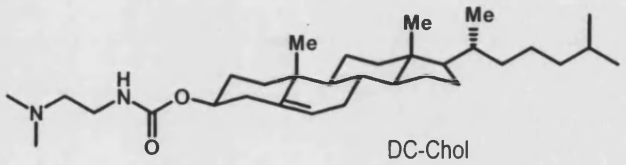


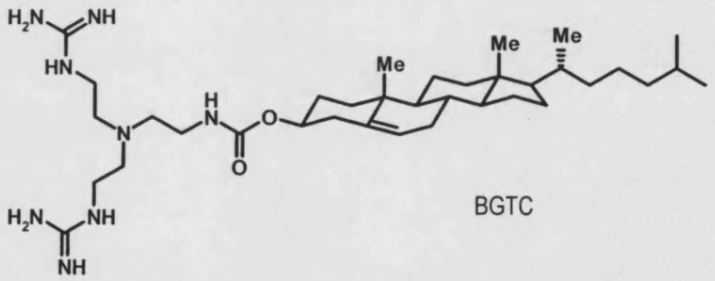
Figure 8. The RPR series of lipopolyamines: linear, globular, T-shaped and branched structures

Cationic lipids II. Steroidal lipids

In 1991, a cationic lipid was synthesised which used the natural steroid cholesterol as the lipid moiety. DC-Chol (Fig. 9) has a single amine functional group, linked to cholesterol as a carbamate (Gao and Huang 1991). DC-Chol/DOPE formulations were shown to have higher activity and lower cytoxicity in the transfection of A431 human epidermoid carcinoma cells than Lipofectin *in vitro*. This prompted other research groups to design cationic lipids using cholesterol as the lipid moiety. In 1996, *bis*-guanidinium-spermine-cholesterol (BGSC) and *bis*-guanidinium-*tren*-cholesterol (BGTC, Fig. 9) were synthesised (Vigneron *et al* 1996). These demonstrate DNA binding due to the high pK_a of the guanidinium functional groups (typically 12.5) and *in vitro* transfection activity comparable to Lipofectin and Transfectam in a variety of cell lines (Pitard *et al* 1999).



DC-Chol



BGTC

Figure 9. Early cholesterol based cationic lipids for gene delivery: DC-Chol and BGTC

A study of synthetic lipopolyamines by the Genzyme Corporation sought to establish structure-activity relationships within a series of polyamines conjugated to cholesterol and other lipid moieties (Lee *et al* 1996). These lipopolyamines and their DNA-complexes have been studied for their potential use in the treatment of cystic fibrosis by transfection of CFT1 (CF airway epithelial) cells *in vitro* and mouse lung *in vivo*.

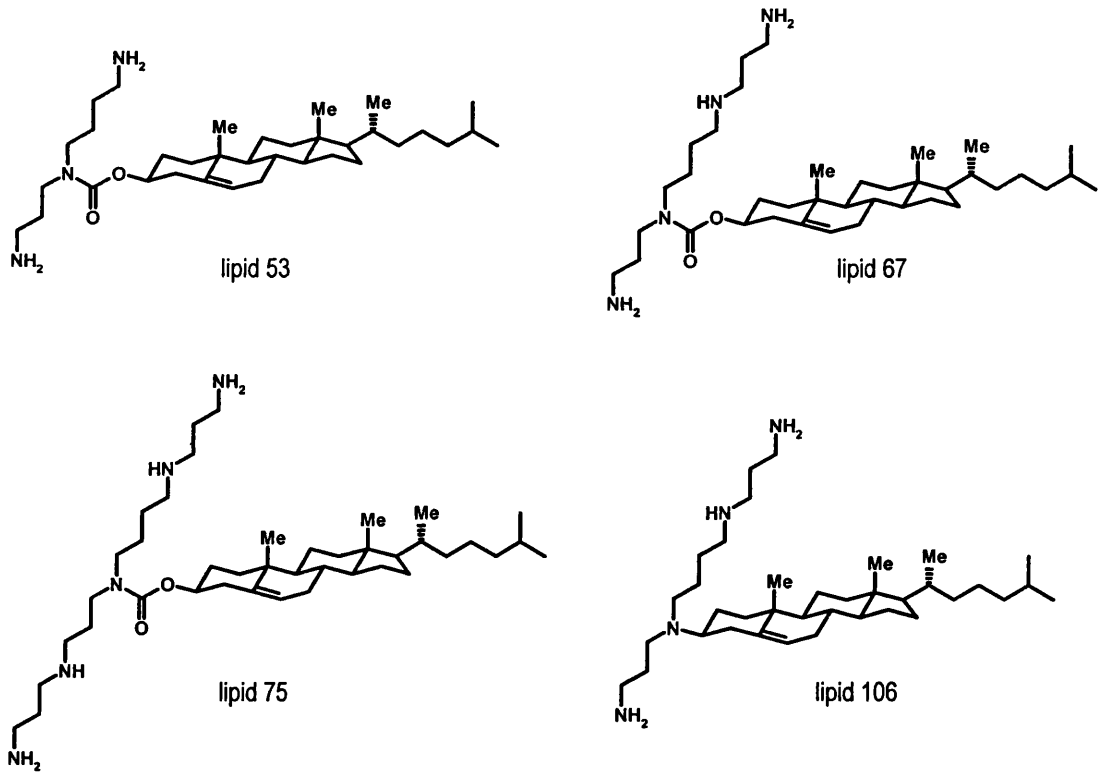


Figure 10. Genzyme lipopolyamines: lipid 53, lipid 67, lipid 75 and lipid 106

In the intranasal delivery of pCF1-βGal (β-galactosidase), pCF1-CFTR (cystic fibrosis transmembrane conductance regulator) and pCF1-CAT (chloramphenicol acetyltransferase) plasmids to the lungs of BALB/c mice (*in vivo*), Genzyme lipid 67, a "T-shaped" cholesteryl carbamate of spermine (Fig. 10) was found to be the most efficacious. "T-shaped" describes polyamines conjugated to lipids along the methylene chain or at a secondary amine. Lipid 67 compared favourably to the spermidine conjugate, lipid 53 (one less amine) and the thermopentamine (3.4.3.3) conjugate lipid 75 (one more amine), showing the optimum number of basic amines in this series to be 3. To explain these results, the authors propose that the spermine headgroup of lipid 67 may have a specific molecular interaction within the mouse lung. They also ascribe the observed decrease in transfection activity of lipid 75 to increased cytotoxic micelle formation as a result of increased water solubility. Also, the regiochemistry of polyamine acylation was shown to be important – linear lipopolyamines (acylated at the

primary amine of the polyamine headgroup) were found to have less activity than their "T-shaped" analogues. The linker is also important – when an amide, amine (lipid 106) or urea replaces the carbamate function, some transfection activity is lost (Lee *et al* 1996).

Formulation with a co-lipid (DOPE) and subsequent optimisation of this formulation was found to give the highest transfection yields and lipid 67 was shown to be the best compound *in vivo*, mediating a 1000-fold higher expression than intranasal delivery of plasmid DNA alone. cAMP-stimulated chloride ion and fluid transport was observed in transfected tissues, functions lost in CF diseased cells. Transgene expression in the mouse was maintained by repeated intranasal administration of lipid 67:DOPE:plasmid DNA complexes. These and results for other compounds in this study did not correlate well with *in vitro* transfection assays. The most active compound *in vitro* was lipid 102, a T-shaped spermine conjugate diether-linked to two C-12 chains (Fig. 11). Despite this, compounds with low activity *in vitro* generally gave low *in vivo* gene expression. Although of some limited use in the biological testing of lipopolyamines for gene delivery, this does not provide a clear rationale for the design of novel efficient non-viral vectors.

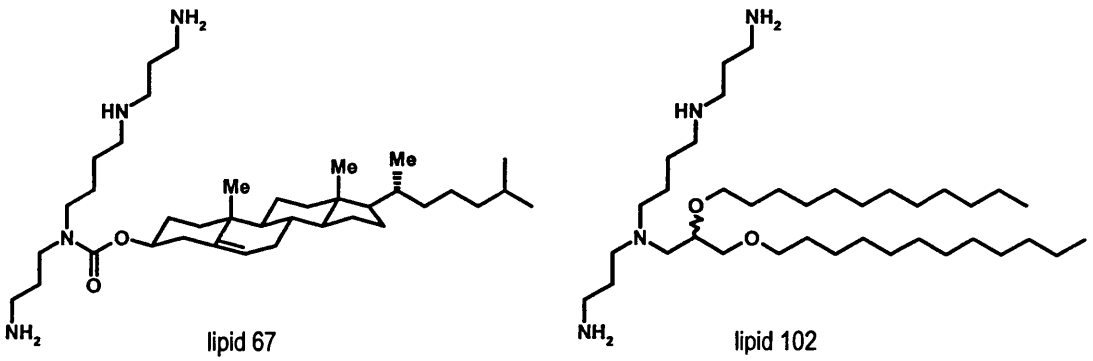
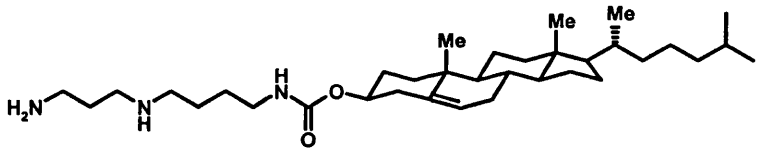
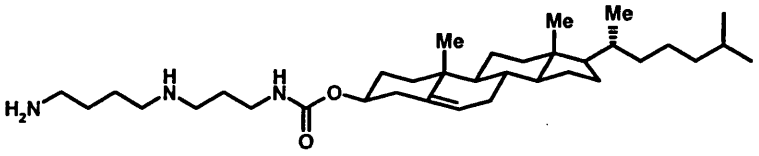


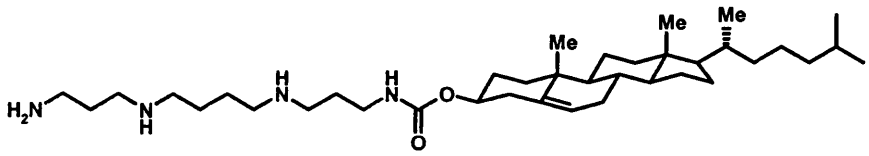
Figure 11. Genzyme lipopolyamines: the most active compounds *in vivo* (lipid 67) and *in vitro* (lipid 102) in their series

In a later independent study, lipid 53 and lipid 67 together with their linear isomeric counterparts (Fig. 12) were used in transfection of myoblasts from dog muscle, a model study for the gene therapy of Duchenne muscular dystrophy (DMD), a human genetic disorder. In optimised formulations with DOPE, these cholesteryl carbamates were compared *in vitro* with canine myoblasts and human lung epithelial cells (A549). Transfection data between these cell lines did not correlate well, lipid 53 and lipid 67 having greater activity than the linear spermidine and spermine derivatives in the myoblast, trends not followed in the A549 cell model. Differences in the cationic-lipid mediated-transfection between cell lines make a comparison of these compounds difficult from the scientific literature as it currently exists, and hence constrains the design of new and more efficient gene delivery tools.



spermidine-cholesterol regioisomers





spermine-cholesterol

Figure 12. Linear lipopolyamine regioisomers of Genzyme's lipid 53 and lipid 67

Genzyme have pursued their studies of polyamine-cholesteryl carbamates by variation of the polyamine headgroup, acylated at a primary amine to afford linear conjugates with "unnatural" methylene spacings between basic amine functional groups (Cooper *et al* 1998). The most active compound *in vivo* was CTAP (Fig. 13), when formulated with DOPE, achieving a similar level of transfection as lipid 67, in the delivery of pCF1-CAT to the lungs of female BALB/c mice. As in studies described previously, the most active compound *in vivo* was not the most active compound *in vitro* (linear thermopentamine conjugate CTAH, Fig. 13). CTAH differs from CTAP in that it contains a terminal 3-aminopropyl moiety rather than a terminal 2-aminoethyl moiety, implying different pK_as and a different overall cationic charge on the polyamine headgroup at physiological pH. Indeed, the authors conclude that methylene spacing in these lipopolyamines is more important than number of amine functional groups in conferring DNA binding strength and ultimately biological activity.

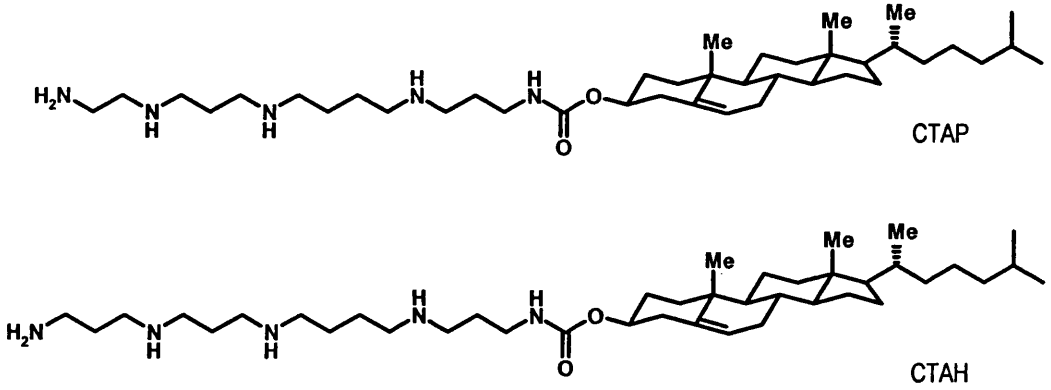


Figure 13. The linear polyamine cholesteryl carbamates CTAP and CTAH

In parallel, linear cholesterol-polyamine carbamates with up to five amino functional groups, including polyethylenimine mimics (Fig. 14) were synthesised (Geall *et al* 1998b). In this series of compounds, pK_a values of these amines were measured and condensation of calf thymus DNA was studied. It was shown that regiochemical distribution of the amines was important in DNA condensation by lipopolyamines and that this is crucial in the design of these compounds for gene delivery.

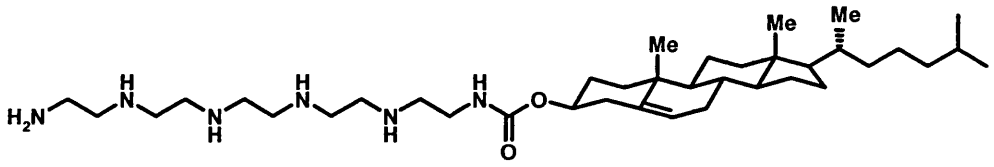


Figure 14. A linear polyamine-cholesteryl carbamate as a lipopolyethylenimine mimic

Since the first cholesteryl carbamates appeared in the literature, lipopolyamines incorporating steroids other than cholesterol have been designed and synthesised for non-viral gene delivery (Geall *et al* 1998a, Walker *et al* 1996, Walker *et al* 1998). The bile acids are a class of naturally occurring steroids, characterised by *cis*-AB ring junctions and mono- to poly-hydroxylation. Polyamines substituted with lithocholic and cholic acid have been designed (Walker *et al* 1996, Geall *et al* 1998a, Blagbrough *et al* 2000). These bile acids have one and three hydroxyl groups respectively, which are present on one side (the α-face) of the steroid skeleton. These polyamine conjugates are therefore facial amphiphiles, with polar and non-polar faces. This is in contrast to the other cationic lipids, which have a polar head and non-polar lipid tail. Polyamine-lithocholic acid amides condensed DNA more efficiently than polyamine-cholic acid conjugates and this effect has been attributed to the higher hydrophobicity of lithocholic acid, carrying only one hydroxyl group (Geall *et al* 1998a). Currently, the best facial amphiphile in this series for *in vitro* gene delivery is a 7,12-bis-glucosylated-cholic acid acylated hexamine, Fig. 15 (Walker *et al* 1996 and 1998). It is reported that weak DNA binding (hence dissociation of DNA from the vector) and an endosome escape mechanism driven by unfavorable interactions between the polar steroid β-face and the lipid bilayer contribute to these molecules transfection activity (Walker *et al* 1998).

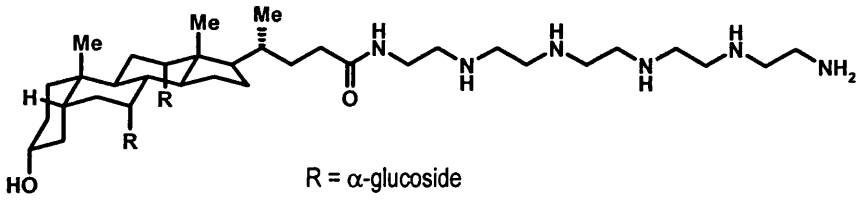
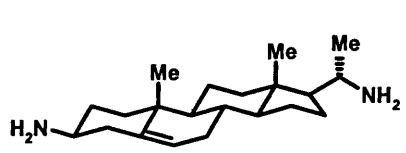
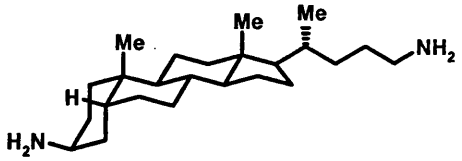


Figure 15. A glucosylated polyamine-cholic acid amide: a cationic facial amphiphile

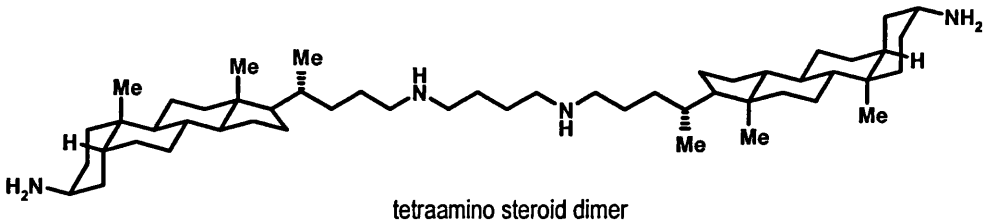
Natural product steroidal amines are known to exert structural control on DNA, including irehdiamine A (Fig. 16), isolated from the West African ireh rubber tree (Hsieh *et al* 1995, Muller *et al* 1995), and polyamine analogues of these compounds have been synthesised incorporating bile acids, for example a tetraamino steroid dimer (Fig. 16) which displays higher affinity for calf thymus and synthetic poly $[d(A\text{-}T)]_2$ and poly $[d(G\text{-}C)]_2$ DNAs than the tetraamine spermine (Hsieh *et al* 1994). These recent studies did not report the potential for these molecules (as cationic lipids) in the delivery of polynucleic acids into cells, however they focused on the binding to and structural manipulation of DNA, pertinent issues in the design of new non-viral transfection agents.



irehdiamine A



synthetic diamino steroid



tetraamino steroid dimer

Figure 16. Natural and synthetic steroidal polyamines: irehdiamine A and synthetic amino steroids

Endosome Escape

Particles of DNA-lipopolyamine (or other non-viral gene delivery vector) complexes are internalised by endocytosis (Miller 1998). At no time during this process does the DNA particle cross a lipid bilayer, indeed endocytosed material is enveloped by a phospholipid bilayer. Therefore, any material contained within the resulting endosome cannot be considered to have entered the cell. Natural cell processes dictate that material contained within an endosome is destined for enzymatic degradation by fusion with a lysosome. In addition to this, in order for the foreign genetic information to be expressed, it must be present in the cell nucleus and therefore must leave (escape) the endosome. Mechanisms for endosome escape vary between non-viral gene delivery vectors. Spontaneous escape may occur in small percentages of DNA-vector containing endosomes (Zabner *et al* 1995), or escape may be induced by addition of chloroquine (Miller 1996, Zelphati and Szoka 1996, Pouton and Seymour 1998). Viruses commonly express peptides on their protein coat which fuse which phospholipid bilayers and cause poration (Moradpour *et al* 1996) and these fusogenic peptides have been investigated for their ability to facilitate endosome escape in non-viral gene delivery systems (Moradpour *et al* 1996, Wyman *et al* 1997, Themis *et al* 1998). Buffering of endosome pH (5.5, Remy *et al* 1994) as a prelude to enzymatic degradation in the lysosome is a natural biochemical process. Fusogenic peptides adopt amphiphilic α-helical conformations around pH 5.0. Such helical structures interact with and disrupt phospholipid bilayers (Morris *et al* 2000). Some non-viral vectors, especially PEI and lipopolyamines containing aminoethyl moieties (e.g. CTAP), possess corresponding pK_a values and are protonated during this process. This protonation drives osmolytic swelling of the endosome, leading to rupture and escape of the endosomal contents into the cytoplasm (Remy *et al* 1998, Godbey *et al* 1999c).

Nuclear translocation

The focus of some current research in the area of non-viral gene delivery has moved away from optimisation of the lipopolyamine vector. Byk, Behr and others (Neves *et al* 1999a, Zanta *et al* 1999) are attempting to increase transfection activity of cationic liposomes by increasing the amount of nucleic acid material that enters the cell nucleus once delivery into the cytoplasm is achieved. Poor nuclear translocation is a hurdle to efficient transfection. Nuclear localisation signals (NLS) are short peptides that mediate movement of large molecules into the cell nucleus (Davis 1999). Typically of 7-12 amino acid residues, most NLS peptides conserve a sequence of five consecutive cationic residues, made up of lysine and arginine (Zanta *et al* 1999). The amino acid sequence PKKKRKVEDPYC is typical of NLS peptides (Zanta *et al* 1999). Some viruses use an NLS in their protein coats to invade the nucleus of a host cell (Davis 1999). PKKKRKV is a conserved sequence in the large tumour antigen of Simian virus 40 (Morris *et al* 2000). Attempts are being made to couple NLS dodecapeptides into a non-viral gene delivery protocol (Ciolina *et al* 1999, Neves *et al* 1999b, Zanta *et al* 1999). Natural and synthetically modified NLS peptides are being covalently coupled to plasmid DNA and oligonucleotides, which are delivered by cationic lipids. Results have thus far been mixed, but this approach has seen success in the HeLa cell line (Zanta *et al* 1999).

Study of transfection by fluorescent microscopy

Thus, although there is now a range of available non-viral agents that promote transfection, still little is known about the mechanisms that underlie this phenomenon. Zabner *et al* (1995) showed that cationic liposomes were taken up by endocytosis yet not all cells expressed genes taken up by this process. Therefore, successful transfection is reliant on efficient intracellular mechanisms and it is important to study this phase of cationic lipid-

mediated gene delivery. Transfection efficiency cannot be improved without a greater knowledge of the molecular detail of intracellular events. Confocal microscopy with appropriate fluorescent molecular probes is now routinely used in the study of intracellular phenomena and is potentially useful for the study of lipopolyamine-mediated transfection.

There are various strategies that may be employed in the confocal imaging of *in vitro* transfection processes. By labelling either the vector or the polynucleic acid (or potentially both with suitably different non-overlapping fluorophores), there is the potential to track the distribution and time course of this distribution of these components in a cell cluster. In the study mentioned above, Zabner *et al* (1995) were the first to attempt direct study of the uptake and subsequent fate of cationic liposomes by fluorescent microscopy. Using DNA covalently labelled with the fluorescent dye ethidium and labelled DMRIE (a monocationic lipid), they observed endocytosis and fusion of endosomes near the nucleus. No fluorescent DNA was detected inside the nucleus and both fluorophores were not detected in the same experiment.

Byk and co-workers have designed a fluorescent probe in the RPR-series of lipopolyamines (Byk *et al* 1998). RPR-121653 (Fig. 17) is a derivative RPR-120535 (Fig. 8), the most efficacious compound in this series. The fluorescent label tetramethylrhodamine isothiocyanate is coupled as a thiourea onto the side chain of lysine, which links the polyamine and lipid functionalities. In fluorescence imaging experiments RPR-120535 has been formulated with RPR-121653 in a 30:1 ratio and incubated with DNA to form fluorescent lipoplexes (Helbling-Leclerc *et al* 1999). Cells were then transfected and the intracellular fate of the lipopolyamine coat followed by confocal microscopy. In their study, the fluorescent lipopolyamine was used to probe differences in myogenic cell uptake of cationic lipids to elucidate the origin of poor transfection *in vivo* compared to results *in vitro*.

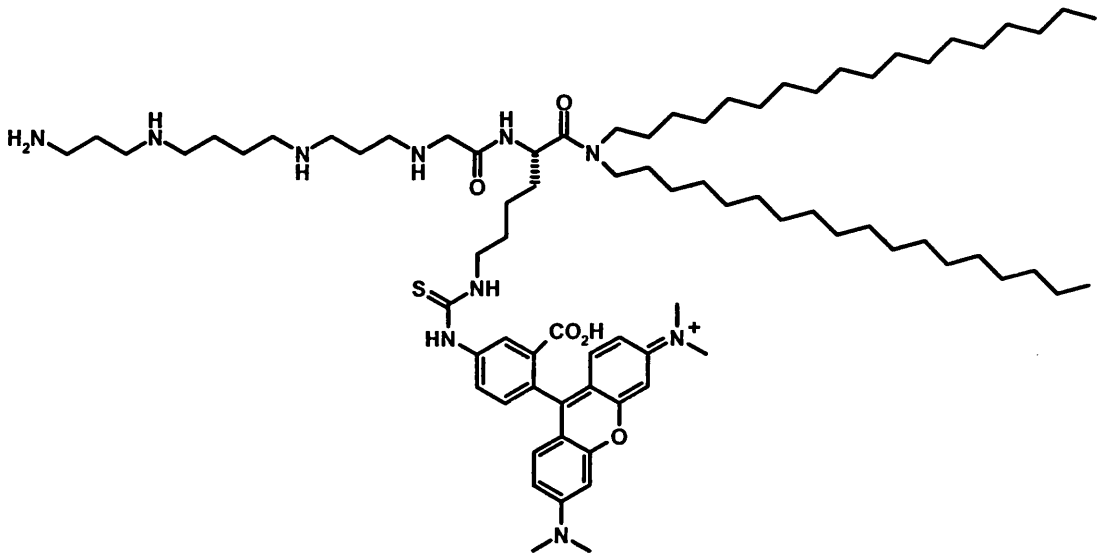


Figure 17. RPR-121653, a tetramethylrhodamine labelled lipopolyamine

DOTAP/DOPE liposomes have also been used in the delivery of antisense oligonucleotides *in vitro* (Marcusson *et al* 1997) for the inhibition of gene expression. For the confocal study of this phenomenon in A549 cells, a fluorescent derivative of DOTAP has been synthesised with a BODIPY fluorophore (Fig. 18). This has been used to deliver rhodamine-labelled 20-mer phosphorothioate oligonucleotides for targeting of protein kinase C-α mRNA. The study demonstrated that the BODIPY label did not affect the ability of the liposome to deliver the antisense DNA to its target sequence in the nucleus. Although short oligonucleotides of this type are not condensed, as are DNA plasmids, there is association with the cationic lipid in the liposome. This association is observed by fluorescence microscopy to break down before the DNA enters the nucleus. By comparing the fluorescence staining of an endosome / lysosome selective probe (TMA-DPH) to the fluorescence pattern observed for the BODIPY-DOTAP conjugate, the authors concluded that these are the final destination for cationic lipids in these cells, although the two fluorophorescent probes were never directly observed to co-localise due to overlap of their emission wavelengths. This limitation highlights

issues in multicolour confocal studies in terms of experiment design, conjugate design and the appropriate choice of fluorophores.

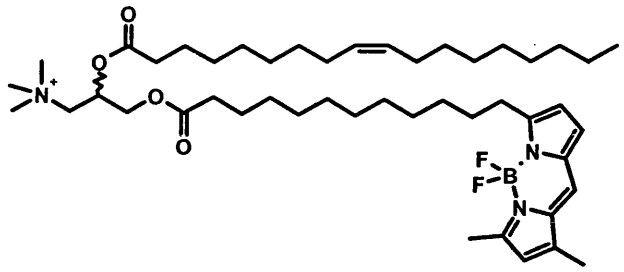


Figure 18. A fluorescent cationic lipid: a BODIPY derivative of DOTAP

As introduced above, the cationic polymer polyethylenimine (PEI) is an efficient DNA condensing agent. This has recently been derivatised with the fluorescent dye Oregon Green 488 (Godbey *et al* 1999b). Labelled PEI was used to condense plasmid DNA labelled with a red fluorophore (rhodamine). The cellular uptake and subsequent fate of this doubly-labelled PEI-DNA complex has been tracked by fluorescent microscopy. The timescale of such events, and the distribution of these fluorophores have been studied. PEI-DNA complexes were seen to enter the cell by endocytosis and the observation of an apparently yellow fluorescence (combination of red and green emission wavelengths) at cell nuclei suggests that PEI and DNA make their way to the nucleus as associated complexes (Godbey *et al* 1999b and 1999c). In their studies, it was also reported that PEI is endocytosed and undergoes nuclear localisation even when administered without nucleic acid material.

Expression of transfected genes may be imaged using a plasmid gene that encodes for a fluorescent protein product or an enzyme that catalyses the formation of fluorescent small molecule products. These genes are known as reporter genes. An example of a fluorescent gene product is the green fluorescent protein (GFP, Cody *et al* 1993). GFP is a natural product from the jellyfish *Aequorea victoria* that undergoes posttranslational modification of three sequential amino acid residues (serine-tyrosine-glycine, S65-Y66-G67). The folding of

the protein promotes cyclisation to an imidazolone followed by an oxidation step to afford conjugation to the phenolic side chain of Y-66 (Fig. 19, Reid and Flynn 1997). The resulting fluorophore is protected from solvent quenching by the surrounding β-sheets and emits visible light at 508 nm upon excitation with UV light. For reviews on the structure, chemistry and use of GFP plasmids as reporter genes, see: McCapra *et al* 1988, Cody *et al* 1993 and Ormö *et al* 1996.

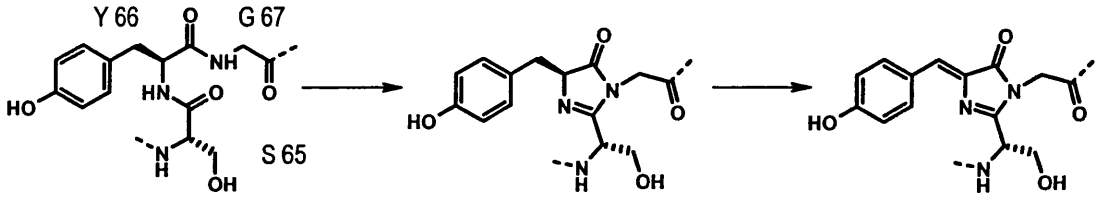
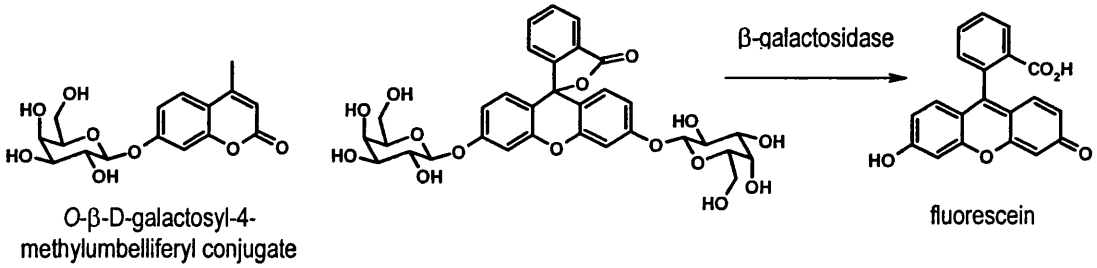


Figure 19. Fluorophore formation in green fluorescent protein

Examples of enzymic gene products are β-galactosidase (β-Gal) and luciferase. These enzymes catalyse the conversion of a non-fluorescent substrate into a fluorescent product. This substrate has to be fed to the cell culture during the transfection experiment. The substrates for the β-galactosidase assay are conjugates of β-D-galactopyranoside sugars where the enzyme catalyses the hydrolysis of β-glycosidic bonds (acetal moieties). A number of substrates are commercially available for this assay, including a 4-methylumbelliferyl conjugate and a fluorescein conjugate (Fig. 20). In this conjugate, the fluorescein is locked into the non-fluorescent spirolactone form by *bis*-*O*-glycosylation with two β-D-galactopyranoside sugars. In the β-galactosidase assay, these are specifically hydrolysed by the newly expressed enzyme to liberate the highly fluorescent fluorescein (Fig. 20).



O-β-D-galactosyl-4-methylumbelliferyl conjugate

Figure 20. Substrates for the β-galactosidase gene expression assay

Aims of this thesis

The aims of this thesis are to understand, design and prepare small molecule lipopolyamines for non-viral gene therapy. Initially, a concise review of the scientific literature, mainly published prior to the start of these synthetic and analytical studies sets the scene for our own medicinal chemistry research in this area of non-viral gene therapy. In order to study the synthesis, analysis and the molecular details of intracellular interactions of lipopolyamine vectors, we outline the design of polyamine-lipid conjugates based on naturally occurring polyammonium and steroid moieties.

The study of the molecular interactions between lipopolyamine gene delivery vectors and polynucleic acids requires an understanding of the synthesis of such designed molecules. Starting from the naturally occurring polyammonium DNA condensing agent spermine, we present the design of its conjugates with the steroidal lipids cholesterol and lithocholic acid. These polyamine-cholesteryl carbamates and polyamine-lithocholic acid amides incorporating alkyl spermidine and spermine polyammonium moieties represent the first synthetic targets, and their interactions with, and condensation of, natural and synthetic DNA will be evaluated.

Non-viral vector mediated cell transfection remains a poorly understood phenomenon both *in vitro* and *in vivo*. In order to study the intracellular distribution and fate of lipopolyamine-DNA complexes we aim to synthesise designed fluorescent models of our synthetic polyamine conjugates of cholesterol and lithocholic acid. Such target fluorescent

lipopolyamines may be considered as analytical tools for the tracking, by confocal microscopy, of lipopolyamine-DNA complexes in cells. Such transfection studies are necessary to observe intracellular events such as endosome escape and nuclear trafficking, two barriers to efficient gene delivery in current non-viral vectored systems. The synthesis of these analytical tools will require chemical modification and manipulation of steroid moieties possessing variation in the configuration at the AB ring junction, and of commercially available fluorophores to produce highly fluorescent lipopolyamine conjugates.

CHAPTER 2

Synthesis and analysis of steroidal lipopolyamines

Synthesis and analysis of steroidal lipopolyamines

Polyamines are distributed extensively in natural biological systems, and along with their alkylated and acylated derivatives, they are involved in a variety of roles such as DNA replication, DNA damage protection, tumour proliferation, ion-channel modification and as components in invertebrate (spider and wasp) venoms. Therefore, syntheses of a wide variety of spermine **1** and spermidine **2** derivatives have been published in the scientific literature (for recent reviews on the roles of polyamine conjugates and their synthesis, see: Blagbrough *et al* 1997, Kariagiannis and Papaioannou 2000, Kuksa *et al* 2000). Initially, our studies of steroidal-polyamine conjugates for non-viral gene delivery focused on the acylation of suitably protected spermine **1** as a route to lipopolyamine carbamates and amides.

The first targets for this work were monoacylated spermine conjugates of the steroids cholesterol **7** and lithocholic acid **17** (Fig. 21) thereby incorporating spermidine **2** as the polyammonium moiety. Fig. 21 illustrates the common structural motifs of our lipopolyamines – a hydrophobic lipid region, which varies in shape about the AB-ring junction, covalently attached to a polyammonium (at physiological pH) DNA-binding region. These hydrophobic and hydrophilic moieties are connected through a linker region built from an amide or carbamate, and this represents a point of disconnection in the synthesis of these molecules.

Many literature syntheses of polyamines build a polyamine chain from smaller units (Pak and Hesse 1998), especially through Michael-additions to acrylonitrile (ethenyl cyanide) and subsequent reduction (of the nitrile functional group) (Bergeron and Garlich 1984). Polyamines and their conjugates may be constructed in this way, or may be derivatised from commercially available polyamines (Blagbrough and Moya 1995, Moya and Blagbrough 1995, Blagbrough *et al* 1996, Geall *et al* 1998b). This may be performed on the solid phase (Byk *et al* 1997, Page *et al* 1998, Carrington *et al* 1999, Wang *et al* 2000).

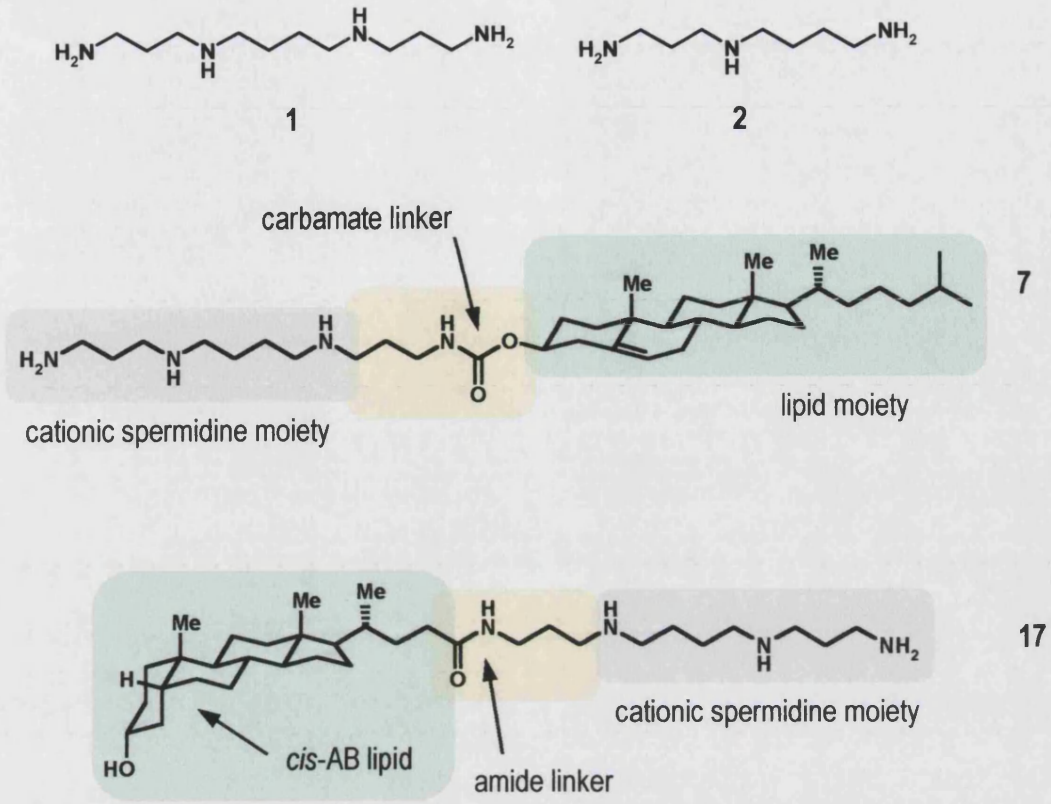


Figure 21. Spermine **1**, spermidine **2** and target steroidal lipopolyamines: cholesteryl carbamate **7** and lithocholic acid amide **17**

For this work we chose a solution synthesis, starting from the readily available spermine **1**. This approach required an efficient monoacylation of this symmetrical tetraamine at a primary amine to afford linear steroidal polyamines. Literature procedures (Ashton *et al* 1995, Blagbrough and Moya 1995, Bischoff *et al* 1997) for the synthesis of such monoacylated polyamine conjugates often rely on the use of large excesses of free polyamine in the presence of the electrophile, and therefore a statistical selectivity for monoacylation of the polyamine, resulting in low yields and the requirement for rigorous chromatographic purification.

Spermine **1** is a symmetrical polyamine with four nucleophilic amines. Therefore, directly acylating this compound with the majority of acylating agents (such as acid chlorides,

anhydrides or chloroformates) yields mixtures of regioisomers (Bischoff *et al* 1997, Bergeron and McManis 1998) of mono-, di-, tri-acylated species along with the tetra-acylated polyamine. In order to address this problem in the synthesis of linear lipospermine derivatives, Blagbrough and Geall (1998) developed a route for the stepwise protection of spermine to afford N^1,N^4,N^9-tri-Boc spermine **5** (Fig. 22). Starting from spermine **1**, a selective protection of one primary amine with ethyl trifluoroacetate affords the trifluoroacetamide **3**. The trifluoroacetyl group is a known protecting group for amines and ethyl trifluoroacetate has been shown to be a useful reagent for the introduction of trifluoroacetyl groups to the primary amines of polyamines (O'Sullivan and Dalrymple 1995, Blagbrough and Geall 1998). Selectivity for acylation or alkylation of primary over secondary amines in polyamines is partly derived from the higher reactivity of primary amines due to steric over electronic effects. However, trifluoroacetamides of secondary amines are known (Pak *et al* 1998, Pak and Hesse 1998). This selectivity in polyamines has been further established by demonstration of 1,3-acyl migrations of trifluoroacetyl groups from secondary amines to newly deprotected primary amines (Pak and Hesse 1998).

In the synthesis published by Blagbrough and Geall (1998, Geall and Blagbrough 2000), the remaining three amines of **3** are Boc-protected, then trifluoroacetamide **4** is cleaved at pH 11 with conc. aq. NH_3 affording primary amine **5**. This route has the convenience of being one-pot in MeOH throughout these three steps and may be performed on a multi-gram scale. Therefore, it was the protection strategy of choice in our synthesis of polyamine-cholesteryl carbamate **7** and lithocholic acid amide **17**.

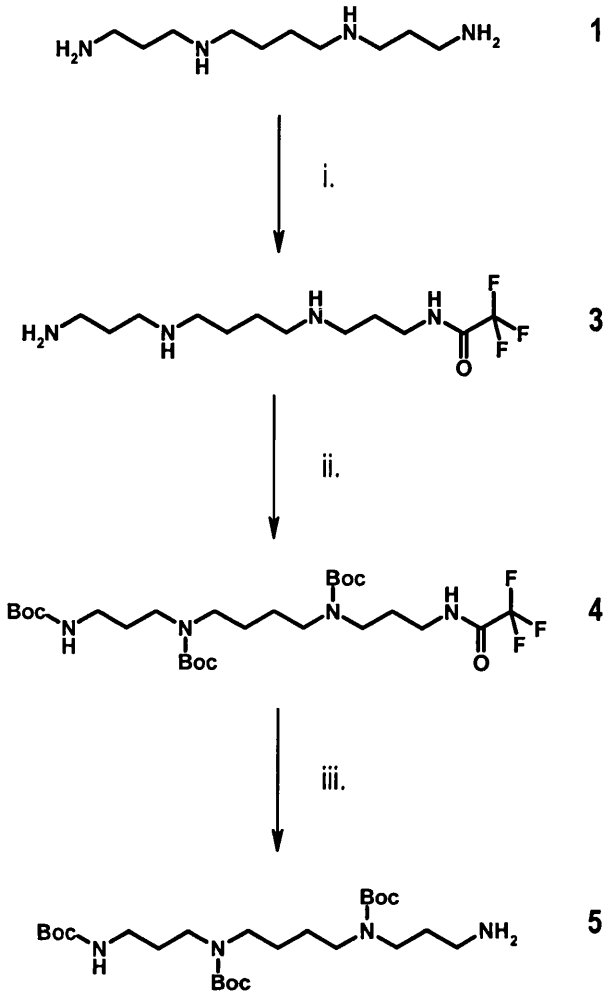


Figure 22. Synthesis of N^1,N^4,N^9-tri-Boc protected spermine **5**

i. CF_3CO_2Et, MeOH, -78 °C to 0 °C over 1 h; ii. $(Boc)_2O$, MeOH, 0 °C to 25 °C over 1 h, 18 h;

iii. Conc. aq. NH_3, MeOH, 25 °C, 18 h

Following this route (Fig. 22), N^1,N^4,N^9-tri-Boc spermine **5** was routinely synthesised on multi-gram scales (typically 3–6 grams of **1** affording typically 4-7 grams of **5**, 45-55 % yield) in 24 h and following only one chromatographic separation from N^4,N^9-di-Boc spermine (derived from an initial di-trifluoroacetylation). Compound **5**, with the appropriate mass spectrum (Fig. 23, FAB-HRMS $C_{34}H_{57}N_2O_2$ requires 503.3809 (MH^+), found 503.3829), displayed fragmentation consistent with the loss of up to three Boc groups, this pattern

(multiple losses of 100 m/z) was reproducible and was regularly observed with the poly-Boc protected polyamines discussed herein.

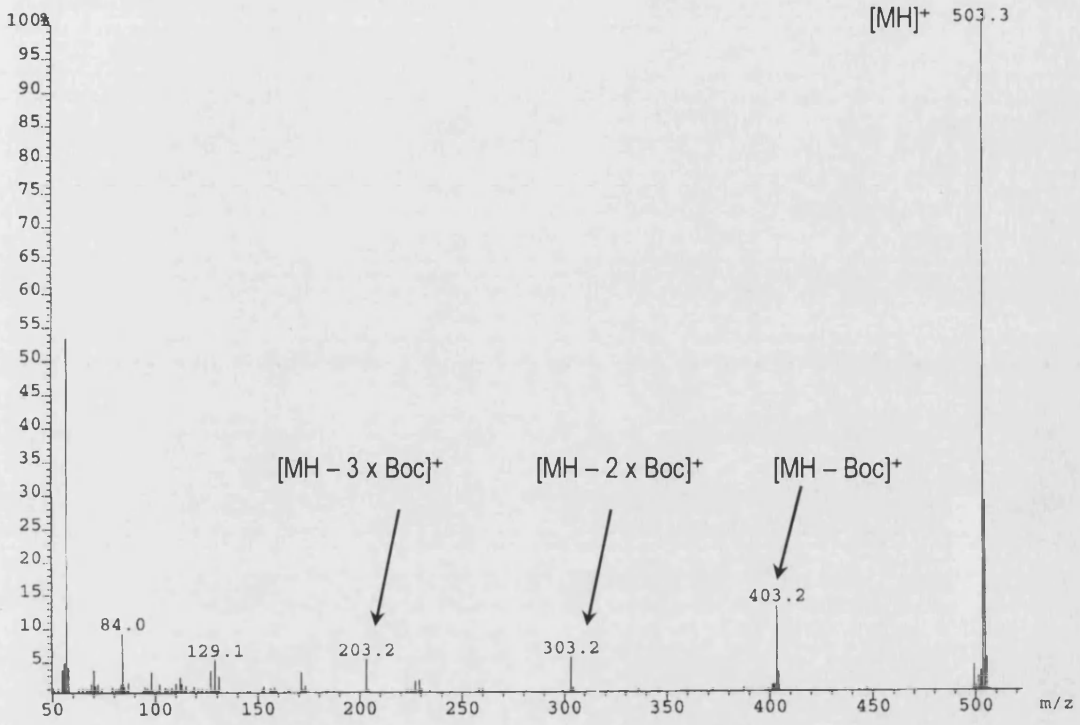


Figure 23. FAB mass spectrum (positive ion mode), showing fragmentation of N^1,N^4,N^9-tri-Boc spermine **5**

Synthesis of polyamine-cholesteryl carbamates

The primary amine of **5** was acylated with cholesteryl chloroformate to afford, after workup and chromatography (EtOAc-hexane 7:3 v/v, R_f 0.65), the carbamate **6** in 86 % yield (Fig. 24). This compound was then treated with TFA in CH_2Cl_2 (1:9 v/v) to effect the Boc-deprotection. The success of this step relies on the cholesteryl carbamate (derived from a secondary alcohol) remaining intact while the Boc carbamates (derived from tertiary alcohols) are deprotected through nitrogen protonation, loss of CO_2 and *iso*-butylene.

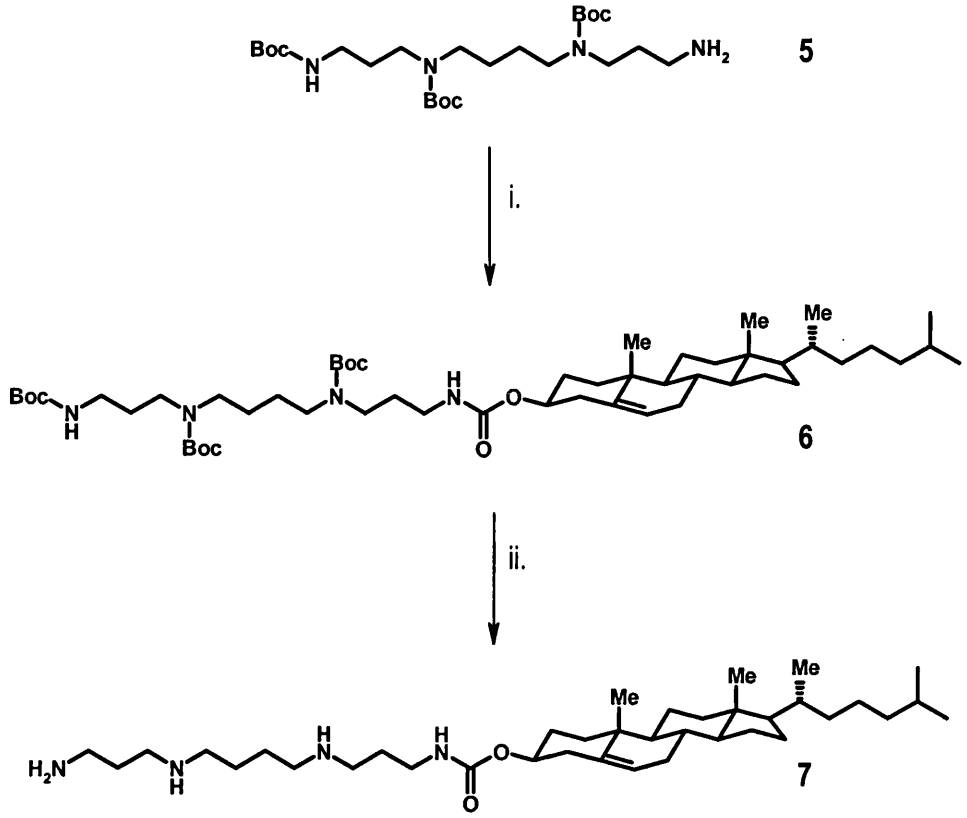


Figure 24. Synthesis of target polyamine cholesteryl carbamate **7**

i. Cholesteryl chloroformate, CH_2Cl_2, TEA, 25 °C, 6 h; ii. TFA-CH_2Cl_2 (1:9), 25 °C, 4 h

These deprotections render polyamines as their poly-TFA salts and hence they are very polar. Even with the use of MeOH and conc. aq. NH_3 in high proportion of the mobile phase, column chromatography gives unsatisfactory results in the purification of these polyvalent (polycationic) compounds. As polyamines are known to be hygroscopic and vary in salt content, melting points and elemental analysis are not reproducible and therefore not particularly relevant techniques for the determination of purity in these instances. Therefore, compound **7** was recrystallised (EtOAc) to provide an analytical sample for NMR analysis, whose spectral data were in agreement with published literature values (Geall *et al* 2000). For DNA work, semi-preparative RP-HPLC (MeCN-0.1 % aq. TFA 6:4 v/v, t_R 7.7 min) was used to purify 10 mg of this material as the poly-TFA salt (99.4 % purity measured by HPLC).

Fig. 25 outlines the numbering system used in the NMR assignment of cholesteryl carbamate **7**. Lithocholic acid polyamine amides are similarly numbered. We have named these target lipids as the parent polyamine and occasionally refer to the abbreviated polymethylene spacing for convenience and comparison. Under this system, spermine **1** is named 1,12-diamino-4,9-diazadodecane (polymethylene spacing 3.4.3) and carbamate **7** is (N^1-cholesteryl-3'-oxycarbonyl)-1,12-diamino-4,9-diazadodecane (3.4.3-Chol).

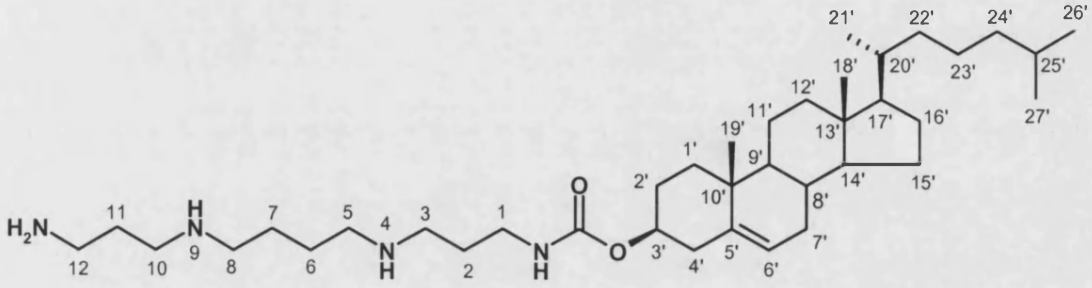


Figure 25. Numbering system for polyamine cholesteryl carbamate **7**

Synthesis of Genzyme lipopolyamines

Polyamine-cholesteryl carbamates such as **7** are lead compounds in the area of non-viral gene delivery, in particular Genzyme's "T-shaped" lipid 67 **10**, where spermine **1** is monoacylated at a secondary amine. This compound is currently in clinical trials for the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene for the gene therapy of cystic fibrosis. In order to improve on literature methods for the preparation of lipid 67 (uncontrolled acylation of spermine **1**, Bischoff *et al* 1997) and to measure pK_a values so that DNA condensation may be compared to our synthesised linear regioisomer **7**, we devised a synthesis using the trifluoroacetyl group to mask selectively both primary amines of spermine (Fig. 26).

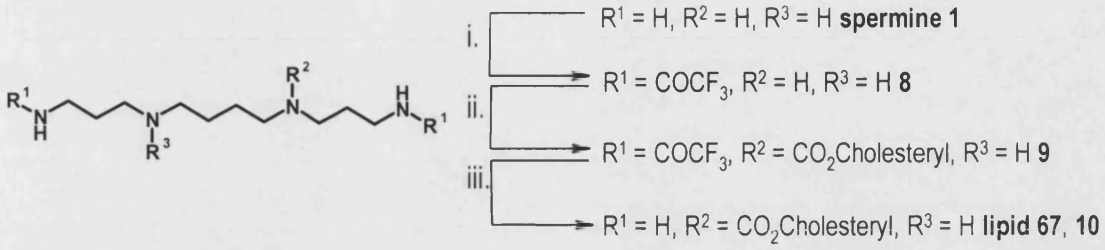


Figure 26. Proposed synthesis of lipid 67 **10**

i. CF_3CO_2Et, MeOH, -78 °C to 0 °C over 1 h; ii. Cholesteryl chloroformate, TEA, CH_2Cl_2, 25 °C; iii. Conc. aq. NH_3, MeOH, 25 °C

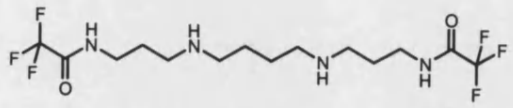
This route was initially attempted as a one-pot procedure, in an analogous manner to the synthesis of **5**. However, the initial trifluoroacetylation proved to be too inefficient for adequate control of the subsequent acylation with cholesteryl chloroformate. The need for MeOH in the final deprotection step limited a one-pot approach in this case, as it is not an appropriate solvent for using cholesteryl chloroformate where there was poor solubility and low nucleophilic reactivity. However, when CH_2Cl_2 was used as the solvent, trifluoroacetylation and acylation with cholesteryl chloroformate proceeded cleanly to afford **9** in good yield (76 %). However, this was only achieved after problematic purification by column chromatography (partial trifluoroacetyl deprotection on the column due to use of NH_3 in the mobile phase (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v, R_f 0.38).

Attempts to deprotect ditrifluoroacetamide **9** using conc. aq. NH_3 in MeOH largely failed due to the poor solubility of **9** in MeOH-H_2O mixtures. Even in solution (requiring large volumes of MeOH, typically 900 ml to dissolve 500 mg of **9**), the deprotection only partially proceeded, leading to crude mixtures of starting material (FAB-MS *m/z* 807 (MH^+), $C_{42}H_{68}N_4O_4F_6$ requires 806) and two monotrifluoracetamides (more polar, ninhydrin positive spots by TLC, FAB-MS *m/z* 711 (MH^+), $C_{40}H_{69}N_4O_3F_3$ requires 710). This method was pursued by bubbling NH_3 gas through neat MeOH at –5 °C, sealing the solution in an airtight

Schott-Duran bottle then heating at 40 °C for 3 days (similar deprotections taking 6 days have been reported in the literature – Doll *et al* 1994). This led to full consumption of starting material **9** and the appearance of a new polar compound by TLC, though attempts to isolate this material failed. The crude material gave the correct mass spectral data for lipid 67 **10** (FAB-MS *m/z* 615 (MH^+), $C_{42}H_{68}N_4O_4F_6$ requires 614; FAB-HRMS calculated 615.5577 (MH^+), found 615.5579). The product gave different chromatography to that of the linear regioisomer **7** (t_R 7.7 min by RP-HPLC, Supelcosil ABZ+Plus, 5 μm, 15 cm x 4.6 mm, MeCN-0.1 % aq. TFA, 6:4 v/v), having less affinity for a reverse phase column (t_R 7.5 min, MeCN-0.1 % aq. TFA, 4:6 v/v), in good agreement with data published by Bischoff *et al* (1997). Disappointingly, this method was not efficient enough for the production of sufficient quantities for further analysis.

During this time, other attempts using literature procedures for the cleavage of trifuoroacetamides employing a variety of bases including K_2CO_3 in MeOH, and trimethylbenzylammonium hydroxide in CH_2Cl_2 (Greene and Wuts 1991, O'Sullivan and Dalrymple 1995, Golding *et al* 1999) also failed. However, a detailed search of the literature revealed deprotections of N^1,N^4,N^{12}-triacylated spermine trifluoroacetamides are known by these reagents (O'Sullivan *et al* 1997).

As part of these synthetic studies in "T-shaped" polyamine-cholesteryl carbamates we acylated spermidine at the secondary amine in an analogous manner to **9** (Fig. 27) to afford ditrifluoroacetyl protected lipid 53 **12**. In repeated small-scale reactions (of upto 40 mg), this product was readily deprotected under a variety of basic conditions (NH_3-MeOH, K_2CO_3-MeOH-H_2O) to give lipid 53 **13** (with the appropriate mass spectral data: FAB-MS *m/z* 558 (MH^+), $C_{35}H_{63}N_3O_2$ requires 557; FAB-HRMS calculated 558.4999 (MH^+), found 558.5010).



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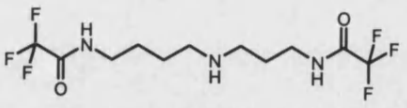

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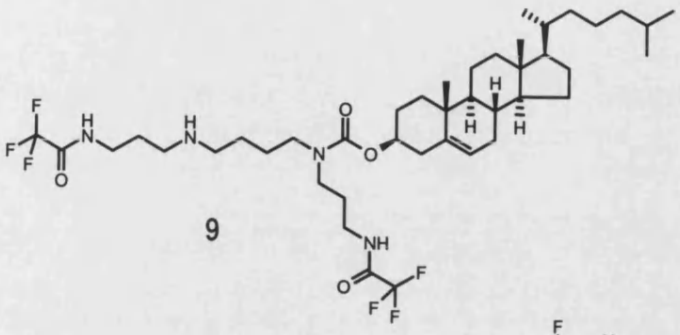

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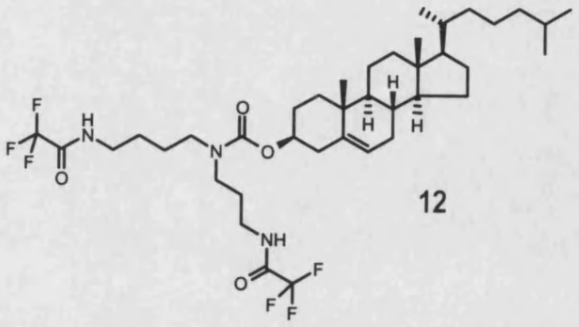

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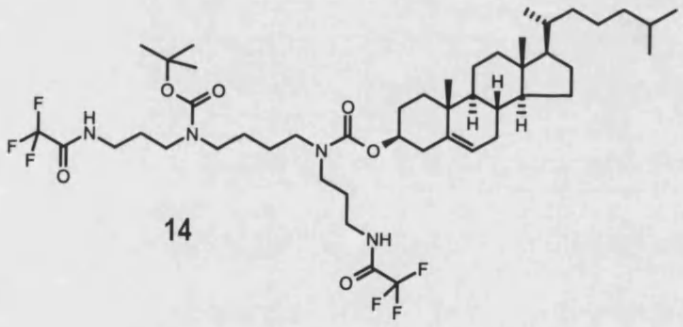

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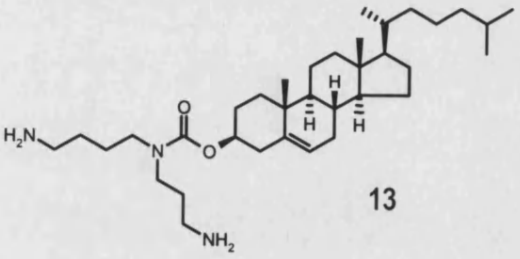

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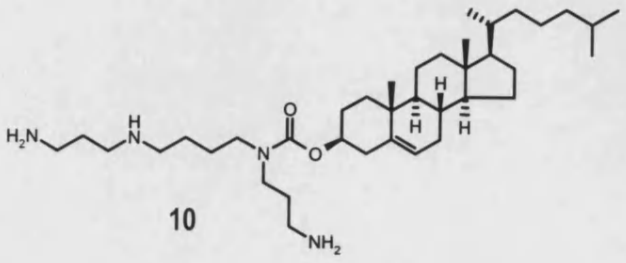

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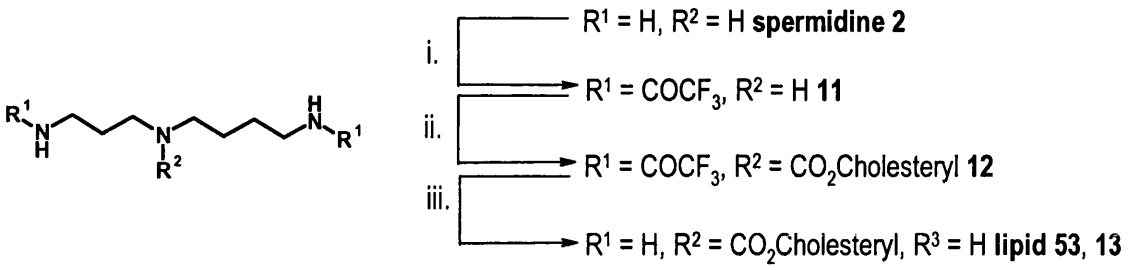


Figure 27. Synthesis of lipid 53 **13**

i. CF_3CO_2Et, CH_2Cl_2, -78 °C to 0 °C over 1 h; ii. Cholesteryl chloroformate, TEA, CH_2Cl_2, 25 °C, 18 h; iii. Conc. aq. NH_3, MeOH, 25 °C, 1 h

This success encouraged us to pursue a modified route to the spermine analogue, lipid 67 **10** (Fig. 28). Starting with the N^1,N^{12}-ditrifluoroacetyl protected spermine **8**, cleanly prepared in high yield (93 %) using the procedures of O'Sullivan and Dalrymple (1995), we acylated the N^4-secondary amine with cholesteryl chloroformate to afford conjugate **9** as previously described. The remaining free amine (N^9) was Boc protected (Boc anhydride in CH_2Cl_2) and the predicted non-polar product **14** was purified by column chromatography (R_f 0.46, CH_2Cl_2-hexane 7:3 v/v). ^1H and ^{13}C NMR data for this compound showed the clear presence of a Boc carbamate (28.8 and 80.9 ppm for Boc methyls and sp^3 quaternary carbons respectively), yet FAB and electrospray mass spectrometry failed to indicate this. Given the predicted chromatography and NMR data, it is likely that the Boc carbamate is particularly labile in compound **14** under the ionisation conditions applied. Due to time constraints we were not able to pursue this point rigorously, but preliminary results show that compound **14** is readily deprotected (MeOH-conc. aq. NH_3) to afford a polar baseline spot by TLC. We argue that compound **15** is readily formed under standard conditions for the cleavage of a trifluoroacetamide (MeOH-conc. aq. NH_3, Greene and Wuts 1991, O'Sullivan and Dalrymple 1995), under which trifluoroacetamides of lipid 67 **10**, such as conjugate **9**, were stable. Also,

after this step, without purification of **15**, the Boc deprotection should be immediately effected (TFA-CH_2Cl_2) in order to purify polyamine conjugate **10** by RP-HPLC.

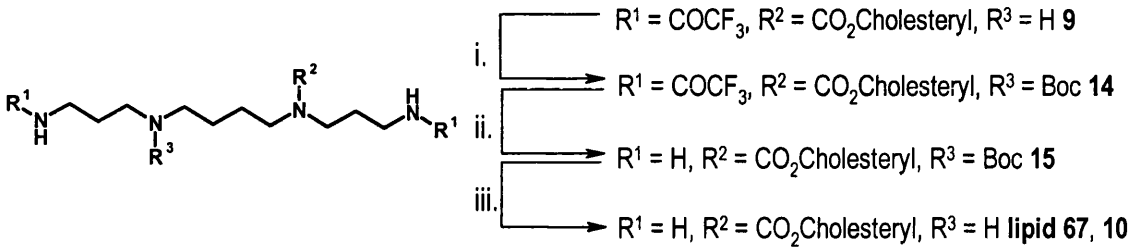


Figure 28. Proposed modified route for the synthesis of lipid 67 **10**

i. Boc_2O, CH_2Cl_2, TEA, 25 °C; ii. Conc. aq. NH_3, MeOH, 25 °C; iii. TFA-CH_2Cl_2 (1:9), 25 °C

Synthesis of polyamine-lithocholic acid amides

The *cis*-AB ring configuration affords bile acids such as lithocholic acid with a different shape compared to the steroid cholesterol which can considered to be planar. Structural differences such as AB ring configuration have been shown to bestow different DNA-binding activities upon amino-steroids (Hsieh *et al* 1995). As discussed in Chapter 1, polyamine amides of lithocholic acid and other bile acids have been shown to condense DNA (Geall *et al* 1998a) and to mediate DNA delivery (Walker *et al* 1996). Spermidine mimics incorporating bile acid moieties are therefore probes of steroid conformation and its influences on DNA binding affinity or transfection efficiency. As part of our synthetic studies of steroidal polyamines, we chose lithocholic acid amides of spermine as target spermidine mimics (Fig. 29).

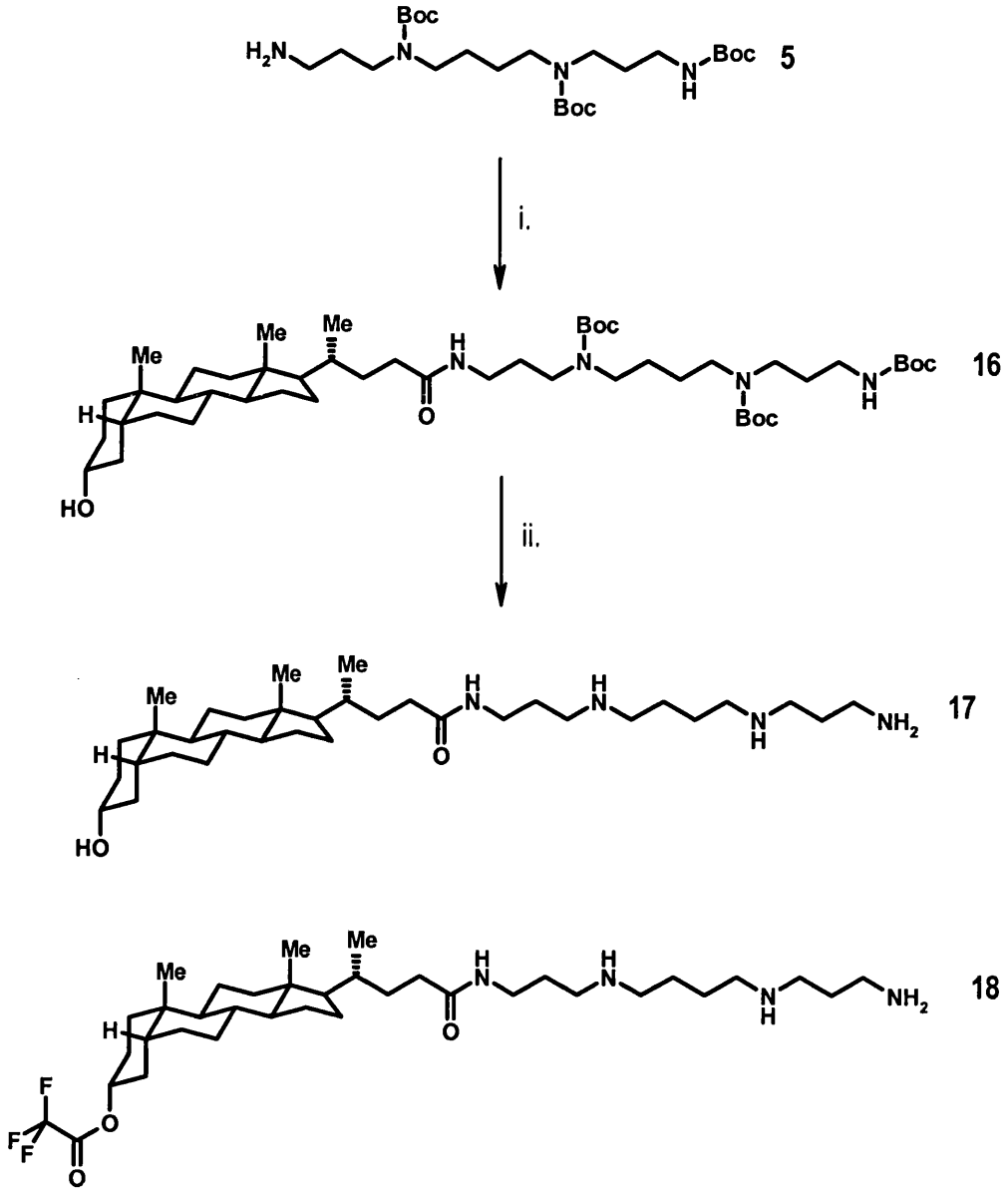


Figure 29. Synthesis of target polyamine-lithocholic acid amide **17**

i. Lithocholic acid, EDC, HOBt, CH_2Cl_2, 25 °C, 16 h; ii. TFA-CH_2Cl_2 (1:9), 0 °C, 4 h

In order to prepare amide **16**, the primary amine of **5** was acylated with lithocholic acid, using the carbodiimide EDC as the dehydrating agent, with a catalytic amount (typically 0.5 molar equivalents) of HOBt. This reaction proceeded smoothly in 86 % yield. Whilst initial attempts to make this compound succeeded, later larger scale preparations suffered from an unclean reaction where the starting acid was not fully consumed. As the product has a similar (higher) R_f to lithocholic acid in a variety of mobile phases, purification proved more difficult.

Washing with base (aq. NaOH) failed to remove this hydrophobic acid. Reaction yields improved when the EDC was added to the suspension of lithocholic acid in anhydrous CH_2Cl_2, followed by HOBt only when the solution cleared (usually after 5 min), indicating the acid had been activated. The amine was added last and this order of addition was used for all future amide (or ester) forming reactions. Where DCC replaced EDC as the coupling reagent, a precipitate of DCU was visible shortly after the addition of the amine (or alcohol for ester formation).

The deprotection of amide **16** proceeded upon treatment with TFA-CH_2Cl_2 (1:9 v/v), however ^1H and ^{13}C NMR data were consistent with the trifluoroacetyl ester **18** of the 3α-hydroxyl group (steroid 3'-CH resonances, values in ppm, for **16** in brackets): ^1H NMR 4.87-4.98 in d_6-DMSO (3.55-3.64 in $CDCl_3$); ^{13}C NMR 79.5 (72.1). The elemental composition provided by the accurate mass spectrum confirmed the ester **18** ($C_{36}H_{64}N_4O_3F_3$ requires 657.4931 (MH^+), found 657.4961).

New hydrophobic moieties in non-viral gene delivery vectors

Transfection efficiency is still too low with published non-viral vectors (Li and Huang 2000, Brown *et al* 2001, Pouton and Seymour 2001, Schatzlein 2001). Together with a need for further understanding the mechanisms underlying gene delivery, we and others are designing new non-viral vectors containing, novel structural motifs. One example of such design is a new class of cationic lipid incorporating a Buckminster fullerene C_{60} core. One hemisphere of the buckyball has been conjugated, via cycloaddition, to a tetraammonium moiety (**19**, Fig. 30, Nakamura *et al* 2000).

GS4 (**20**, Fig. 30) is a cationic *gemini* surfactant with two C_{12}-lipid chains conjugated to four lysine residues, affording up to six positive charges at physiological pH. This compound has some *in vitro* transfection activity when used alone, below its critical micelle concentration.

When used in formulation with the zwitterionic "helper" lipid dioleoylphosphatidylethanolamine (DOPE) and a basic polypeptide, transfection activity is enhanced. Novel spermine-based cationic gemini surfactants symmetrically acylated with two C_{18}-lipid and two tri-lysine chains, with up to 8 positive charges, have also recently been reported (Ronsin *et al* 2001).

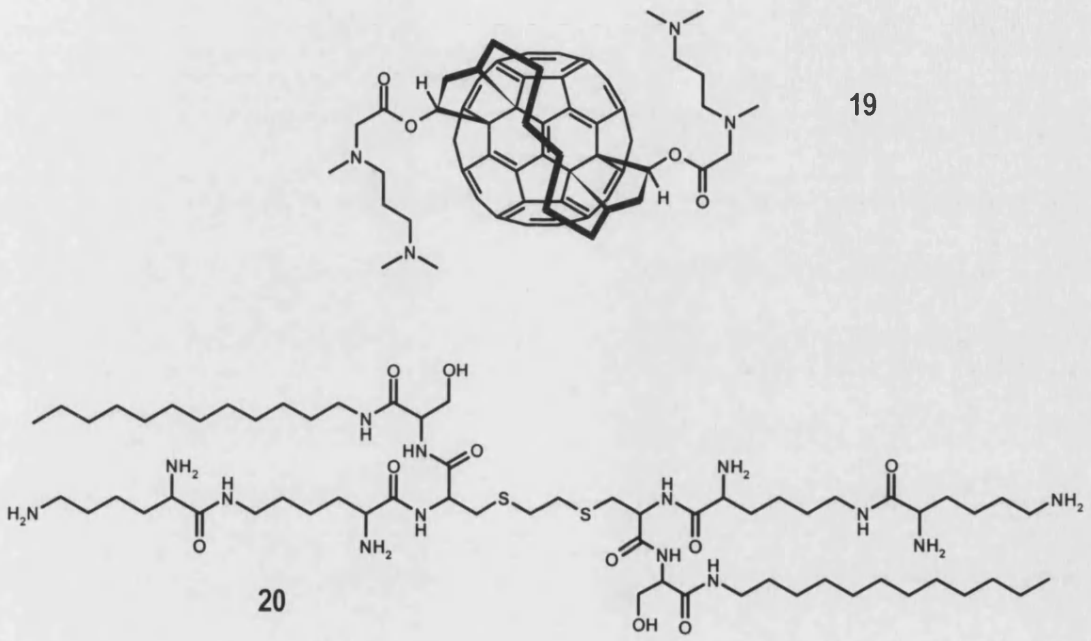
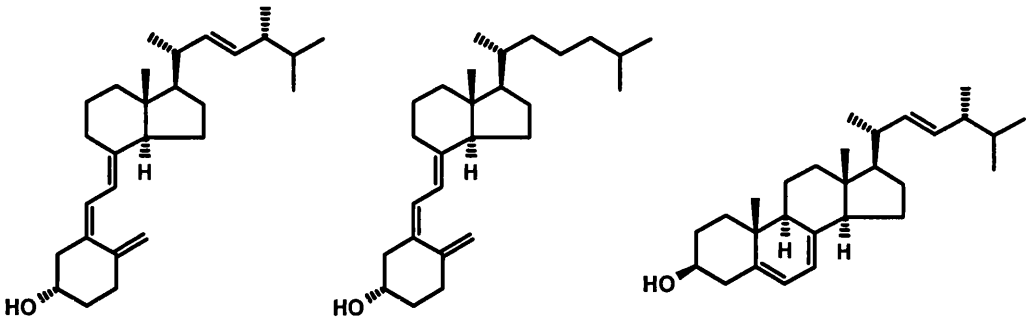


Figure 30. Polyamine-Buckminster fullerene conjugate **19** and GS4, a cationic *gemini* surfactant **20**

During our synthetic studies in lipopolyamine-mediated DNA condensation, new cationic lipids for gene delivery have been designed incorporating the D-vitamins as the lipid moieties (Ren *et al* 2000). Vitamin D_2 (ergocalciferol, **21**, Fig. 31) and vitamin D_3 (cholecalciferol, **22**, Fig. 31) are steroids possessing an open B-ring, formed by UV irradiation of the biochemical precursors ergosterol (**23**, Fig. 31) and 7-dehydrocholesterol respectively (Curino *et al* 1998), promoting an electrocyclic ring-opening followed by a thermal sigmatropic [1,7]-H shift.



21 22 23

Figure 31. Structures of vitamin D_2 **21**, vitamin D_3 **22** and ergosterol **23**, the biosynthetic precursor of vitamin D_3

Ren *et al* (2000) designed and synthesised carbamates of vitamin D_2 and vitamin D_3 incorporating primary, tertiary (**24**, Fig. 32), quaternary and poly- (**25**, Fig. 32) amine moieties as the cationic headgroups. Vitamin D_3 conjugate **24** carries a dimethylaminoethyl carbamate moiety which, at physiological pH, mimics the one positive charge possessed by 3β-[*N*,-(*N*',*N*''-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol, **26**, Fig. 32, Gao and Huang 1991), the first steroid (cholesterol)-based cationic lipid for gene delivery. The polyamine-vitamin D carbamates are monoacylated triethylenetetraamine (*tren*) conjugates (e.g. carbamate **25**, Fig. 32) and are therefore small molecule mimics of branched polyethylenimine (see Chapter 1). The authors of this study do not detail pK_a values for carbamate **25**, but it is likely these basic moieties are dications (not trications) at physiological pH due to the presence of two β-aminoethylamine groups. These *tren*-based steroidal polyamines are similar to second generation cholesterol-carbamates such as *bis*-guanidium-*tren*-cholesterol BGTC (**27**, Fig. 32, Vigneron *et al* 1996, Pitard *et al* 1999). However, the charge of a protonated guanidine moiety is delocalised over four atoms, therefore the regiochemical distribution of positive charges at any given time is not neccesarily the same in molecules **25** and **27**. Despite guanidines having high pK_as and high binding affinity for DNA (explained by net charge neutralisation and also

hydrogen bonding, Vigneron *et al* 1996), the precise interactions of these *bis*-guanidine cationic lipids are unclear. A comparison of the structures of these cholesterol and vitamin D based cationic lipids follows (Fig. 32).

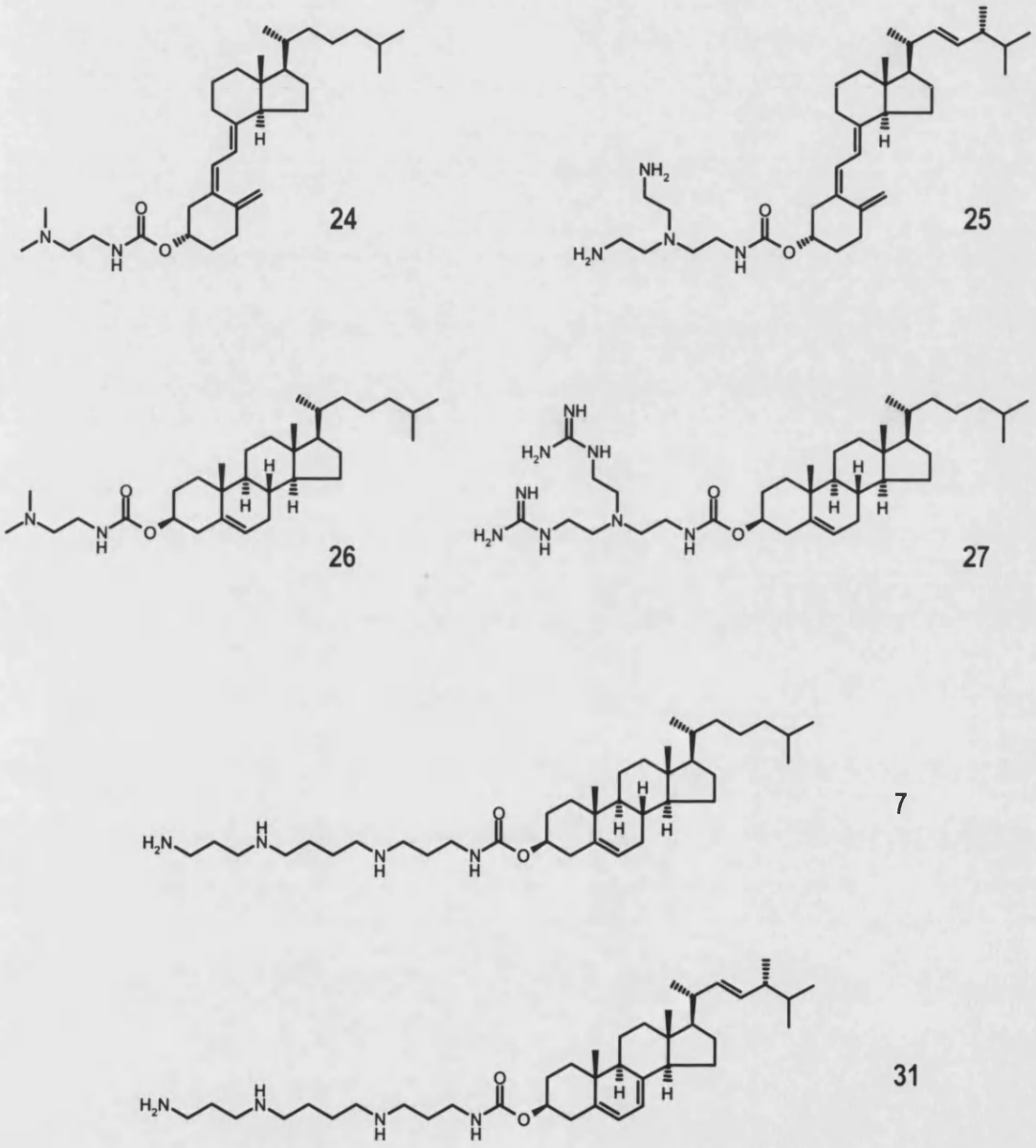


Figure 32. Structures of vitamin D-based cationic lipids (**24** and **25**) and related cholesteryl carbamates (DC-Chol **26**, BGTC **27** and **7**) and target polyamine ergosteryl carbamate **31**

Polyamine-carbamates of vitamins D_2 and D_3 were shown to be the most active in the transfection of BL-6 murine melanoma cells with luciferase plasmid over a wide a range of lipid:DNA charge ratios (lipids were 1:1 formulation with the co-lipid DOPE). Therefore, we have designed the novel steroidal polyamine carbamate **31** (Fig. 32), incorporating ergosterol **23** (Fig. 31) as the steroid moiety. Using our readily prepared N^1,N^4,N^9-tri-Boc spermine **5** we aimed to couple a polyamine moiety to ergosterol using 1,1-carbonyldiimidazole (CDI) coupling (Totleben *et al* 1997, Ren *et al* 2000) in order to introduce an alkyl spermidine polyammonium moiety bearing 2.4 positive charges at pH 7.4 (using pK_a values inferred from cholesteryl carbamate **7**, Geall *et al* 1998b and 1999).

Initially, our proposed synthesis of carbamate **31** (Fig. 33) followed the route of Ren *et al* (2000), using CDI to couple amine **5** to ergosterol **23** via an introduced carbonyl (carbamate) group. In their syntheses of vitamin D analogues of steroidal amines (including **26** and **27** carboxylic ester. This chloroformate equivalent (cf. our synthesis of cholesteryl carbamate **7** using the readily available chloroformate) was then stirred at 25 °C with two molar equivalents of the appropriate commercially available amine. In order to validate this procedure, a model carbamate, 6-benzyloxycarbonylaminohexanyloxy-*N*-carbonylpropylamine **28** (Fig. 33), was prepared from primary alcohol **41** (described later in this chapter) and 1-propylamine (1.1 equivalents) in 64 % yield with the appropriate ^{1}H and ^{13}C NMR spectra and FAB accurate mass data (FAB-HRMS $C_{18}H_{29}N_2O_4$ requires 337.2127 (MH^+), found 337.2131).

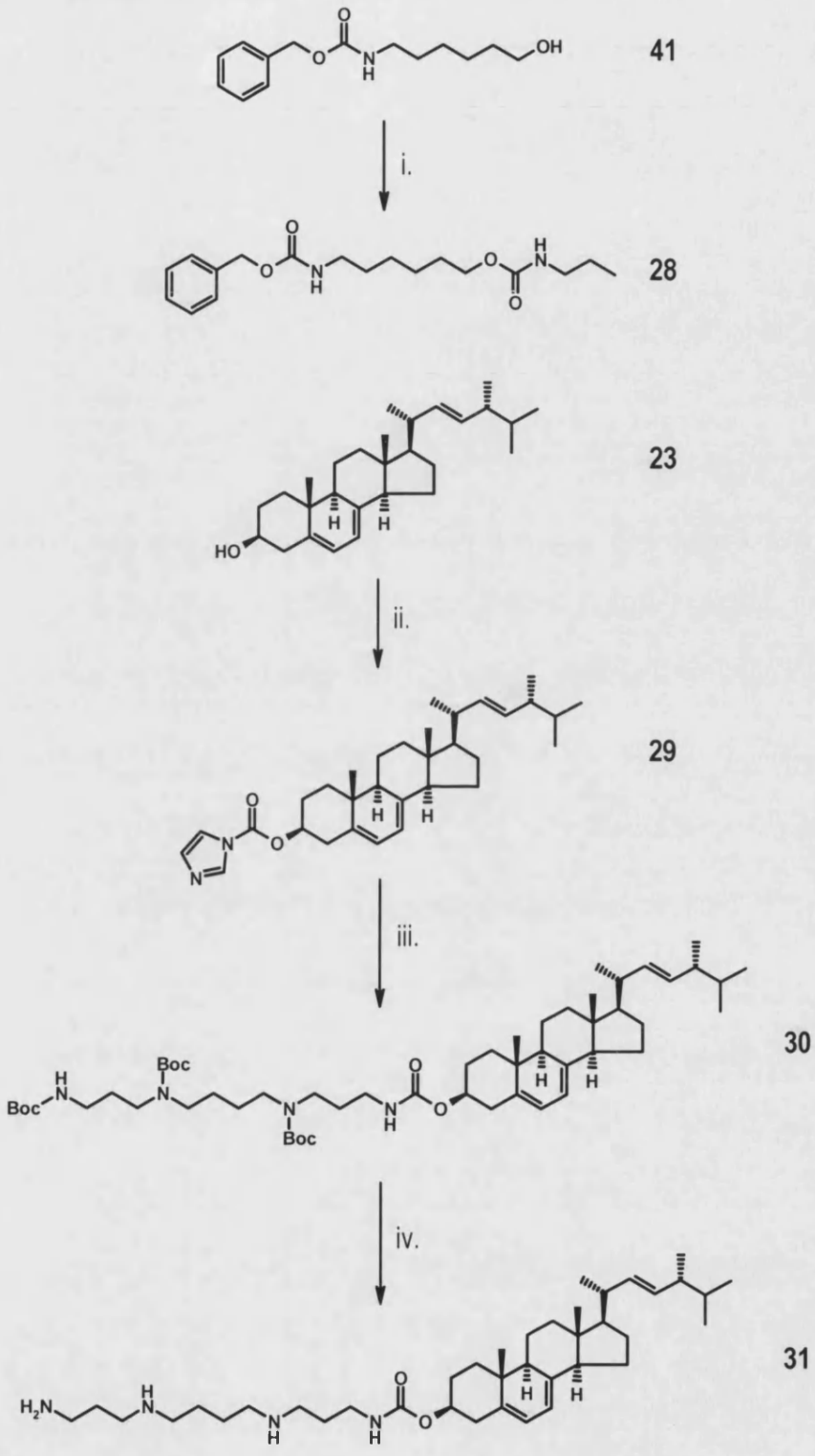


Figure 33. CDI chemistry: synthesis of **28** and proposed synthesis of target ergosteryl carbamate **31**

i. CDI, TEA, CH_2Cl_2 reflux then 1-propylamine, 25 °C, 2 h;

ii. CDI, TEA, CH_2Cl_2 reflux; iii. **5**, CH_2Cl_2, 25 °C; iv. TFA-CH_2Cl_2 (1:9), 25 °C

In our hands, this method proved unsatisfactory for the synthesis of tri-Boc protected ergosteryl carbamate **31**. Subsequent to the addition of one equivalent of amine **5**, a product less polar than ergosterol was observed which stained with ninhydrin when monitored by TLC. This product was isolated by column chromatography (EtOAc mobile phase) and was identified as the imidazole carboxylic ester intermediate **29** by the presence of aromatic signals in the ^{1}H NMR (8.12 (1 H, d, *J* = 1.17, N-CH-N), 7.41 (1 H, dd, *J* = 1.56, 1.17, CH-CH-NCO-O), 7.05 (1 H, d, *J* = 1.56, CH-CH-NCO-O)) and the 3'α proton in resonance at 4.87-4.95 ppm. In separate procedures this pure compound was reacted with amine **5**, yet all attempts to prepare target carbamate **30** (including use of elevated temperatures) failed using this route.

In a model reaction, intermediate **29** was stirred with a large excess (more than 10 equivalents) of *N,N*-dimethylamino-3-propylamine to afford carbamate **33** (Fig. 34). This ergosteryl carbamate is a dimethylaminopropyl mimic of the first steroidal cationic lipid in the area of non-viral gene therapy, DC-Chol **26** (Fig. 34, Gao and Huang 1991) which incorporates cholesterol as the steroid moiety.

In an attempt to provide an efficient route to ergosteryl carbamates, via a more reactive intermediate, we sought to make first ergosteryl chloroformate **32**. Chloroformates are common reagents for the synthesis of carbamates and carbonates, in reaction with amines and alcohols respectively and are invariably made through the reaction of the relevant alcohol and the highly toxic phosgene (Cotarca *et al* 1996). In order to make ergosteryl chloroformate, we attempted to use the safer "phosgene reagent" triphosgene (for a comprehensive review of triphosgene chemistry, see: Cotarca *et al* 1996). Triphosgene (bis-(trichloromethyl) carbonate) is a crystalline solid at room temperature (therefore easy to handle) and reacts with nucleophiles in a manner consistent with three molecules of phosgene and has therefore been used in the preparation of isocyanates and chloroformates (Cotarca *et al* 1996). In our hands, triphosgene was unsuitable for the rapid and efficient conversion of ergosterol into its

chloroformate (less polar, when monitored by TLC). When an excess of phosgene in toluene (20 % w/v solution) was used in accordance with literature procedures (Reed *et al* 1995) we observed quantitative conversion of the alcohol to the chloroformate **32**. Reaction of **32** rapidly afforded carbamate **33** upon reaction with *N,N*-dimethylamino-3-propylamine (analytical data comparable to the previously synthesised sample). Ergosteryl chloroformate was successfully reacted with polyamine **5** to afford carbamate **30** in 60 % yield, displaying comparable chromatography and spectroscopy to the poly-Boc-protected cholesteryl carbamate **30** (steroid 3'-CH resonances, values in ppm for **6** in brackets): ^{1}H NMR 4.52-4.64 (4.43-4.53); ^{13}C NMR 73.1 (73.9). For DNA binding experiments (Chapter 4), carbamate **33** was lyophilised as its trifluoroacetate salt.

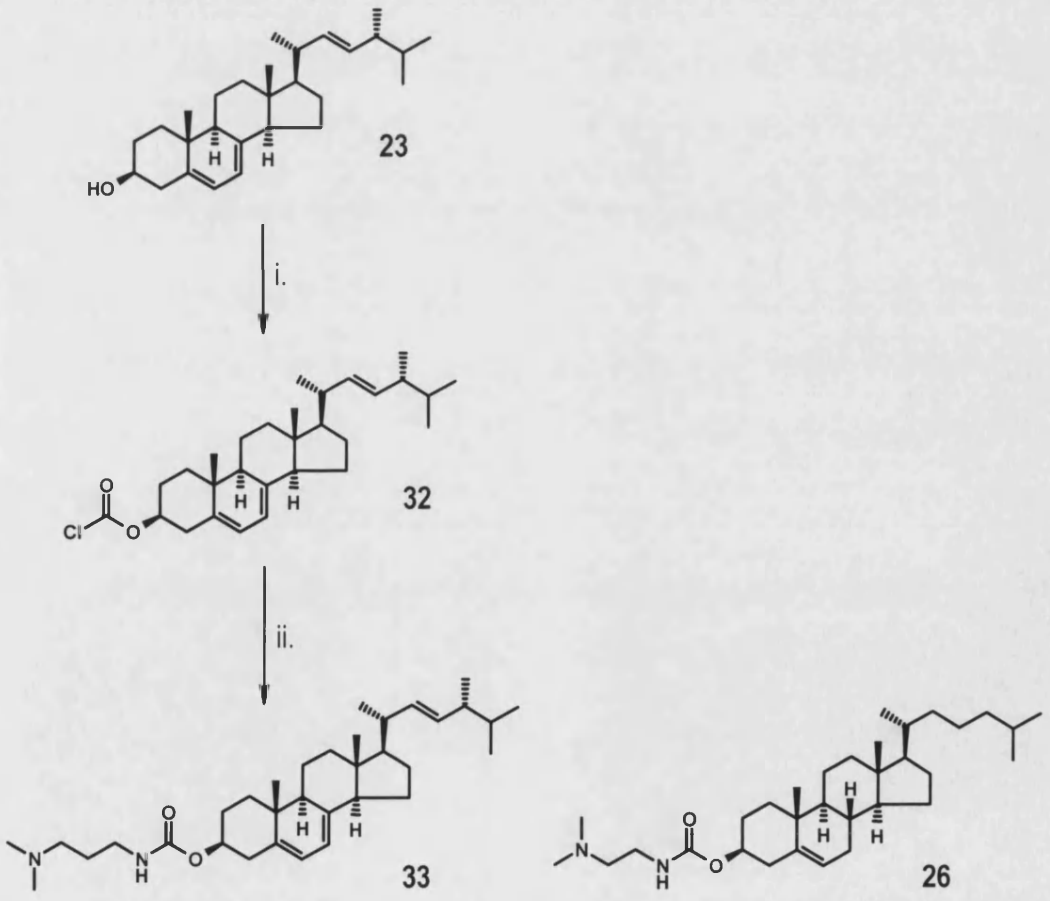


Figure 34. Synthesis of ergosteryl carbamate **33** (cf. DC-Chol **26**)

i. $COCl_2$ (20 % in PhMe), THF, 25 °C, 2 h; ii. *N,N*-dimethylamino-3-propylamine, CH_2Cl_2, 25 °C, 1 h

Synthesis of steroidal spermine mimics – retaining the net cationic charge of spermine

The acylated spermine derivatives synthesised here are spermidine mimics in that they retain the 3.4-methylene spacing between ammonium moieties. The pK_as of cholesteryl carbamate **7** are 10.1, 8.6 and 7.3 (potentiometrically measured, Geall *et al* 1998b), affording our steroidal-polyamine conjugates a net positive charge of 2.4 at physiological pH (according to the Henderson-Hasselbach equation, Geall *et al* 1998b, 1999 and 2000). DNA binding and condensation are sensitive to the number and distribution of positive charges (Geall *et al* 1998b, Geall *et al* 2000 and Chapter 4). Spermine, the natural DNA condensing polyamine has a net positive charge of 3.8 at pH 7.4 (Geall *et al* 1998b). Therefore, we aimed to increase the number of positive charges (from 2.4) on our steroidal polyamines to mimic the charge and cation distribution of spermine.

Reduction of polyamine-lithocholic acid amides

Of the many procedures for amine synthesis, reduction of an amide is established in polyamine chemistry (Lane 1976, Kuksa *et al* 2000, Wang *et al* 2000). The reintroduction of a fourth pK_a from acylated spermine moieties may be achieved by this procedure. We investigated the reduction of lithocholic acid amides of spermine with borane reagents. Muller *et al* (1996) synthesised steroidal-tetraamines such as **37** (Fig. 35) through the reduction of diacylated putrescine (1,4-diaminobutane) with borane-THF. The steroid dimer **37** is a tetraamine with two hydrophobic *cis*-AB steroid moieties. In DNA binding studies, compound **37** displayed higher binding affinities for calf thymus, poly[d(G-C)]$_2$ and poly[d(A-T)]$_2$ sequences than analogous single steroid triamines and simple polyamines such as spermine (Hsieh *et al* 1995, Muller *et al* 1996). The polymethylene spacing in compound **37** is greater than in natural polyammonium ions such as spermine. In order to probe the DNA binding of symmetrical steroidal spermine derivatives and to provide a model for the reduction of

polyamine amides for the retention of pK_a in polyammonium moieties, we designed target molecule **36**.

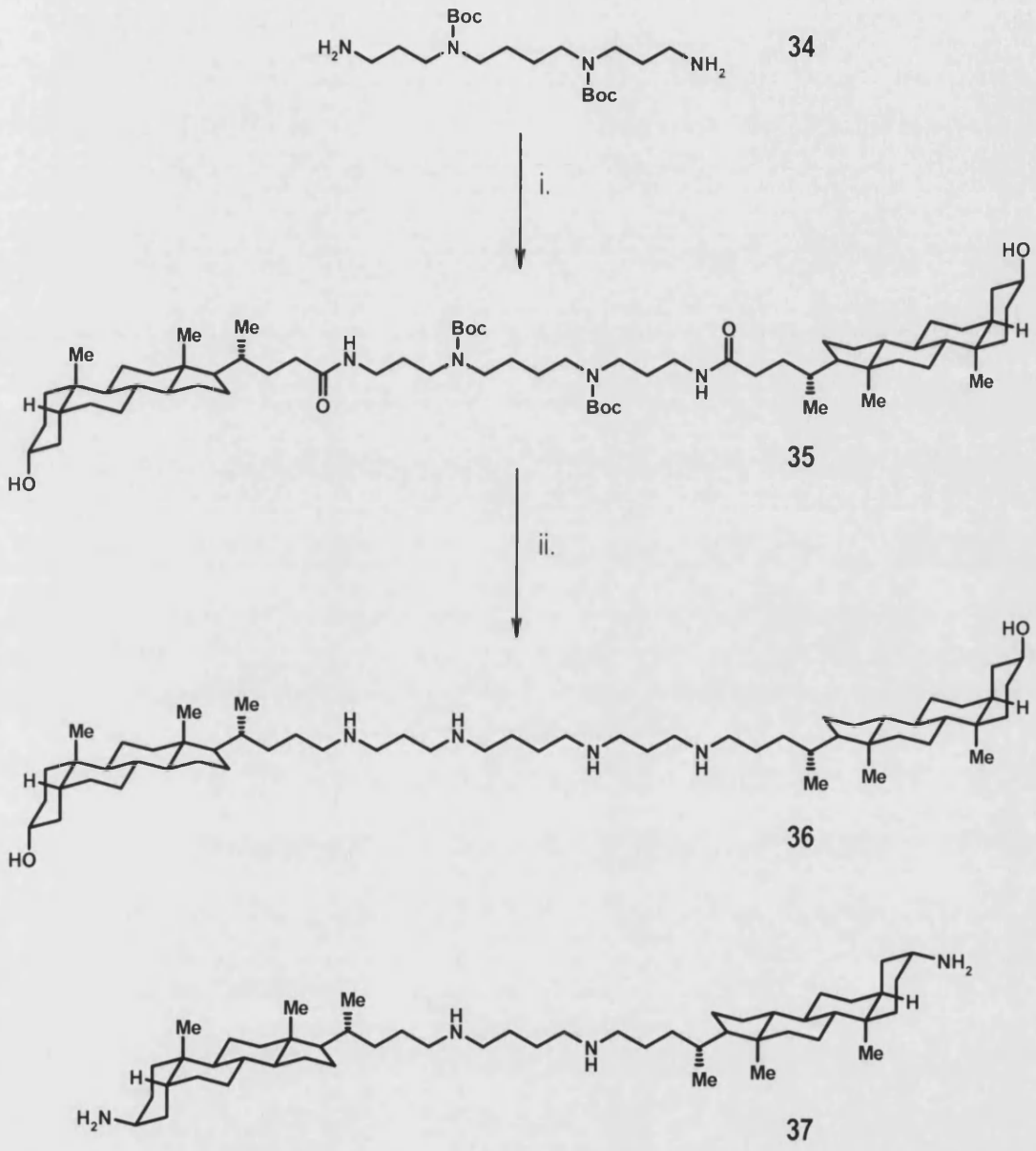


Figure 35. Synthesis of dialkylated lipospermine **36** and the structure of tetraamino-steroid **37**

i. Lithocholic acid, EDC, HOBt, CH_2Cl_2, 25 °C, 16 h; ii. TFA-CH_2Cl_2 (1:9), 25 °C, 2 h; iii. BH_3.DMS, THF, reflux, 12 h then dil. aq. HCl, THF, reflux, 2 h

The di-*N*-terminally acylated spermine derivative **35** was synthesised by analogous chemistry to the mono-acylated poly-Boc proteced polyamine **16**. Di-Boc spermine **34** was synthesised from N^1,N^{12}-ditrifluoroacetylated spermine **8**. Preliminary studies showed the Boc carbamates were sensitive to borane reduction. Instead of attempting a selective reduction of amide **35**, we followed the procedures of Hsieh *et al* (1995) and effected a Boc-deprotection prior to treatment of the amide with a reducing agent. Reduction of both amides was achieved with borane-DMS after Boc-deprotection to afford the symmetrically dialkylated spermine conjugate **36**. This spermine derivative is the first compound in the area of non-viral gene delivery designed with two steroid moieties. However, this compound was insoluble in a range of solvents, and only weakly soluble in d_6-DMSO for analysis by ^{1}H NMR. Also, this compound, despite possessing two hydroxyl functional groups and existing as the poly-hydrochloride salt was not water soluble and therefore was unsuitable for assessment of its interactions with calf thymus or synthetic DNA. Assuming further manipulation of the salt form will increase water solubility of the polyamine (for example, making the poly-nitrate salt instead of the poly-hydrochloride), knowledge of the pK_a values of this spermine derivative is essential to assess DNA binding as a function of polyamine/DNA charge ratio.

This reduction of polyamine amides is an efficient route for the incorporation of polyammonium moieties into steroidal lipopolyamine gene delivery vectors, mimicking the charge and charge distribution of spermine for efficient DNA condensation. This amide reduction approach is readily applied to the bile acid amide-based lipopolyamines, but an alternative route to cholesteryl carbamate-based spermine mimics is required.

Unsymmetrical polyamine extension by reductive amination

We sought to synthesise lipophilic spermine polyammonium DNA condensing agents by alkylation of the polyamine prior to derivatisation with steroids. In order to effect a regio-controlled unsymmetrical extension of spermine, we chose a reductive alkylation strategy (Fig. 36). This methodology has been established in this group (Blagbrough and Geall 1998, Geall and Blagbrough 2000). The synthesis conveniently starts with the previously described N^1,N^4,N^9-tri-Boc spermine **5** and proceeds by alkylation of the primary amine with the appropriate Z-protected aldehyde. Z is orthogonal to Boc (each protecting group may be added/removed in the presence of the other), and Z may be deprotected by a palladium catalysed hydrogenation, which is our chemistry of choice over other hydrogenation methods involving acetic acid which might deprotect Boc groups.

The synthesis of tetra-Boc protected *N*-alkyl spermine derivatives began with the desymmetrisation of spermine to yield N^1,N^4,N^9-tri-Boc spermine **5** as previously described. In parallel, 3-aminopropan-1-ol **38** and 6-aminohexan-1-ol **39** were treated with an excess of benzyl chloroformate in DCM with triethylamine (to scavenge any HCl byproduct), to yield the respective Z-protected ω-amino alcohols **40** and **41**, which were isolated from any traces of benzyl chloroformate, by column chromatography to give both products, typically in 80-90 % yield.

These Z-protected amino alcohols were oxidised to their respective aldehydes with activated DMSO (Swern conditions: DMSO/oxalyl chloride, triethylamine work-up; Mancuso *et al* 1978, for a review, see: Marx and Tidwell 1984). The desired aldehydes **42** and **43** were obtained under these conditions, with full consumption of the starting alcohols observed after 30 min in almost all attempts. These reactions were typically performed on a 1.0–2.5 gram scale, in around 60 ml solvent. Although formation of acetals is known under these conditions (Tidwell 1990), in our hands these side-reactions were not observed.

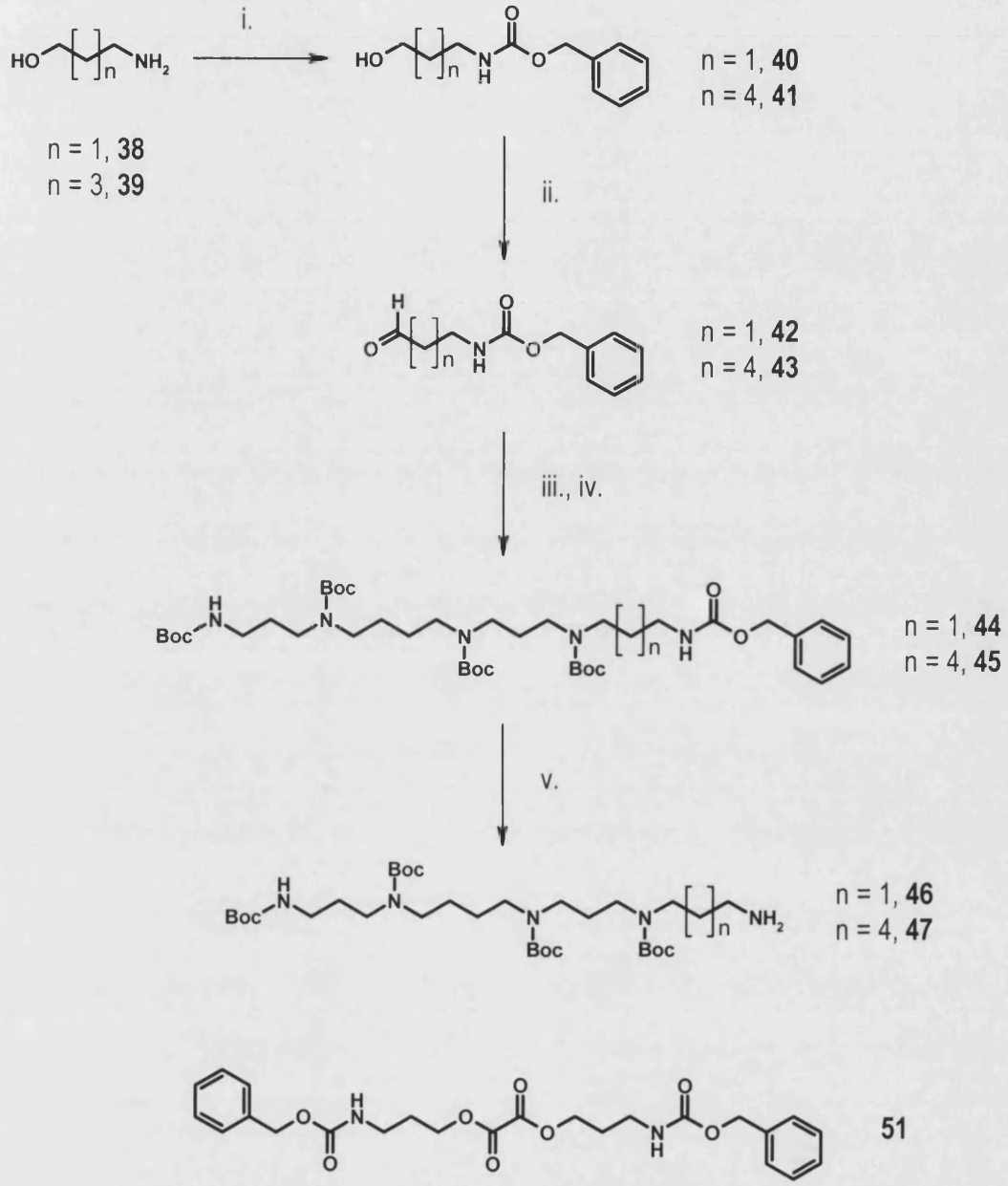


Figure 36. Synthesis of *N*-alkyl spermine derivatives **46** and **47** by reductive amination of aldehydes **42** and **43** i. Benzyl chloroformate, TEA, CH_2Cl_2, 25 °C, 4-18 h; ii. Oxalyl chloride, DMSO, CH_2Cl_2, -78 °C, 30 min, then TEA 0 °C; iii. **5**, $NaBH_3CN$, cat. glacial AcOH, MeOH, 25 °C, 24 h; iv. $(Boc)_2O$, CH_2Cl_2, 25 °C, 3 h; v. Pearlman's catalyst ($Pd(OH)_2$, 20 % on carbon), H_2, MeOH, 25 °C, 18 h

The target aldehydes were separable from their starting alcohols (by TLC, EtOAc/hexane) and initially confirmed as new aldehydes by the formation of 2,4-dinitrophenylhydrazones on treatment with acidic 2,4-dinitrophenylhydrazine (2,4-DNP) solution. After purification (silica gel chromatography), these aldehydes **42** and **43** were confirmed by (data for **42**) ^{1}H NMR (9.78 ppm) ^{13}C NMR (201.3 ppm) and IR (1713 cm^{-1}). DMSO and DMS were common contaminants, rendering these products oils.

These Swern oxidations generally proceeded smoothly and cleanly (60-70 % yield) provided solvent and all reagents were rigorously dried. In our hands, the use of wet DMSO in the reaction of primary alcohol **40** gave a clean product less polar than the starting material by TLC, with the same *R*f value (0.52, EtOAc-hexane 8:2 v/v) as the desired aldehyde. In this instance, the product spot did not stain with 2,4-DNP. Although formation of the corresponding acetals was suspected, purification (silica gel) and subsequent characterisation ($^{1}H/^{13}C$ NMR and FAB^{+} MS) identified the product as the Z-protected alkyl diester of oxalic acid **51**. This reproducible result was observed for both the propanol **40** and hexanol **41** derivatives.

The reaction between amines and aldehydes is in equilibrium with the corresponding imine and water, therefore drying of the solvent and reagents and inclusion of molecular sieves to remove water *in situ* were employed in order to drive the reaction of polyamine **5** with aldehydes **42** and **43** in the desired direction. However, this equilibrium is believed to result in the modest yields obtained and the need for rigorous purification after these and subsequent steps. These reductive alkylations of tri-Boc spermine were performed at pH 5, with catalytic addition of glacial acetic acid. At this pH, sodium cyanoborohydride reduces iminium species faster than aldehydes, again a driving force for imine formation as iminium is irreversibly removed from the system. Some reduction of aldehydes **42** and **43** was observed and this also may have contributed to lower yields. These reactions were stopped typically after 24-48 hours, and following difficult purification over silica gel, the secondary amine intermediates

were obtained with significant, but not unmanageable quantities of primary amine and Z-protected amino alcohol. The new secondary amine was then Boc-protected with an excess of Boc anhydride in CH_2Cl_2. Without purification (though an analytical sample of **45** was purified by silica gel column chromatography, FAB-HRMS $C_{44}H_{78}N_5O_{10}$ requires 836.5749 (MH^+), found 836.5747), the Z-protected terminal amines **44** and **45** were deprotected by hydrogenation (Pearlman's catalyst/H_2). Prior to this step the reaction mixture was treated with conc. aq. ammonia in MeOH to quench any excess of Boc anhydride as substitution of Z for Boc carbamates during hydrogenation in the presence of Boc anhydride is known (Greene and Wuts 1991). The resulting unsymmetrically homologated poly Boc-protected polyamines 3.4.3.3 (thermopentamine) **46** and 3.4.3.6 **47** were purified (silica gel) from tetra-Boc spermine and the respective amino alcohols.

Synthesis of steroidal spermine mimics

Tetra-Boc protected polyamines **46** and **47** were coupled to steroids of our choice. Acylation with cholesteryl chloroformate in CH_2Cl_2 and tertiary base (Fig. 37) gave the respective polyamine-cholesteryl carbamates **48** and **49** in high yields (typically 61-95 %).

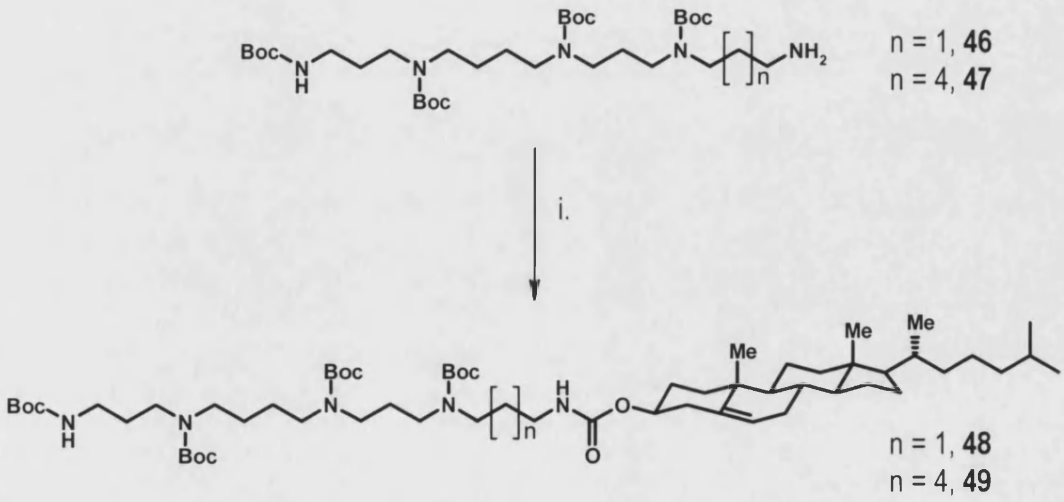


Figure 37. Synthesis of poly-Boc protected cholesteryl carbamates **48** and **49**

i. Cholesteryl chloroformate, TEA, CH_2Cl_2, 25 °C, 18 h

In order to synthesise lithocholic acid amide **50**, the acid was reacted with an excess of EDC and catalytic HOBt, followed by the addition of amine **46** (Fig. 38). As discussed earlier in this chapter, pre-activation of the acid in anhydrous solvents was found to give the fastest reaction times and highest yields.

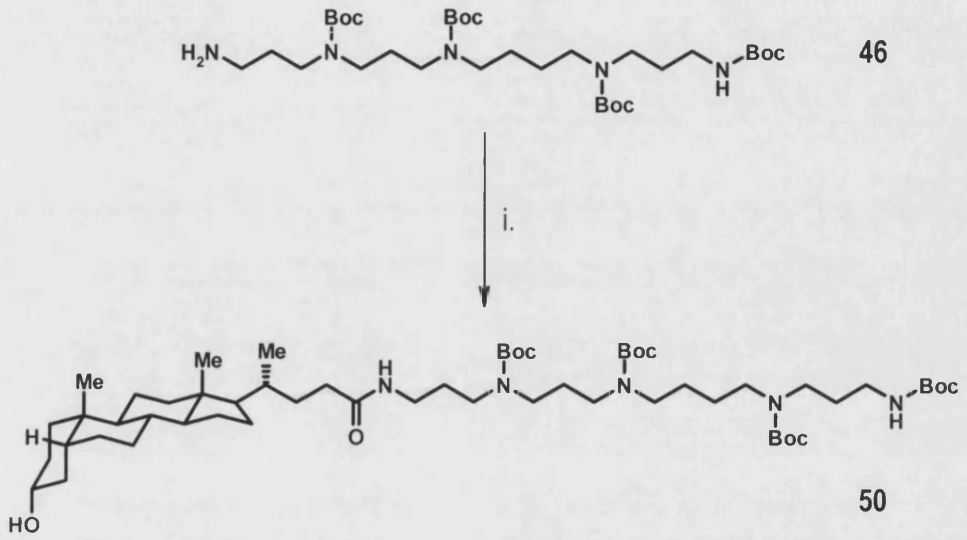


Figure 38. Synthesis of poly-Boc protected lithocholic acid amide **50**

i. Lithocholic acid, EDC, HOBt, CH_2Cl_2, 25 °C, 16 h

In this chapter, the synthesis of polyamine-steroid conjugates in the context of non-viral gene delivery has been discussed. These conjugates of cholesterol and lithocholic acid incorporating Boc-protected spermidine and spermine moieties are precursors to lipopolyammonium DNA condensing agents and their fluorescent derivatives, the design and synthesis of which is discussed in Chapter 3.

CHAPTER 3

Synthesis of fluorescent steroidal lipopolyamines:

Magic markers for the transfection process

Synthesis of magic markers for the transfection process

Non-viral gene therapy vectors achieve the delivery of packaged foreign DNA into target cells, but barriers inside the cell limit the degree of expression of these newly introduced genes. Transfection efficiency cannot be improved without a greater knowledge of the molecular detail of intracellular events. Using such detail as a rational starting point for the design of new gene delivery vectors may aid the realisation of a clinically useful non-viral approach to gene therapy. As discussed in Chapter 1, fluorescence-based confocal techniques are useful for the microscopic study of transfection at the cellular level. This requires the design and synthesis of new analytical tools for the accurate determination of the interactions and processes mediated by such vectors. Herein we continue our studies in steroidal lipopolyamines through the design and synthesis of steroid-based fluorescent gene delivery vectors.

Initial targets were based on polyamine amides of lithocholic acid and cholesteryl carbamates, the synthesis of which was discussed in Chapter 2. Lithocholic acid is characteristic of bile acids which have *cis*-AB ring junctions, but also possess a 3α-hydroxyl group, which we rationalised might be suitable for functionalisation with fluorescent moieties (Fig. 39). The alcohol functional group might not be ideal, being a secondary alcohol (rather than a primary), but it is sterically accessible, on the lower (α) face of the steroid. 3β-Cholesteryl carbamates however, lack even such suitable functionality for the preparation of analogous fluorescent compounds (Fig. 39).

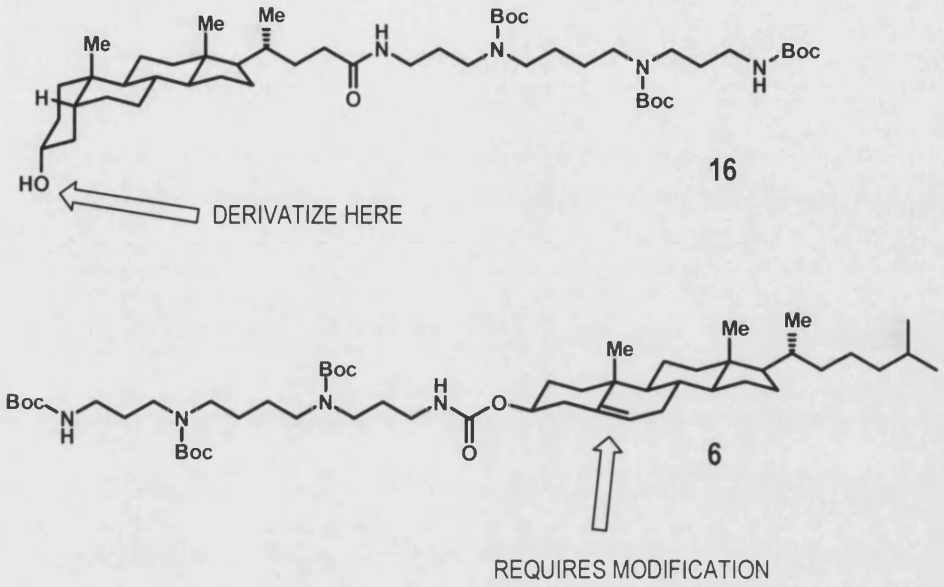


Figure 39. Points of modification of protected lithocholic acid amide **16** and cholesteryl carbamate **6** for the design of fluorescent conjugates

In order to solve this problem and to enable the synthesis of fluorescent steroidal polyamines, we chose a modification of the Δ^5-alkene of cholesterol in order to furnish the steroid with a hydroxyl group so that analogous treatment of planar and *cis*-AB hydroxylated steroids (sterols) may be carried out. Such a hydration of cholesterol should be regio- and stereochemically controlled. There are many ways of introducing oxygen functionality to alkenes and a detailed search of the literature revealed cholesteryl alkene oxidations by epoxidation (Holland and Kahn 1985) and hydroboration (Nussim *et al* 1964a, Tavares *et al* 1993, Izzo *et al* 1998). These hydroborations of cholesteryl and of other steroidal alkenes (Boynton and Hanson 1995, Hanson *et al* 1995, Kuhl *et al* 1999) exemplify an attractive route which we will use to access hydrated cholesterols in one step (cf. an epoxidation strategy) with a degree of regio- and stereoselectivity.

In order to hydroborate cholesteryl carbamate **6**, we selected and then modified the procedures of Tavares *et al* (1993) who reported difficulties during their hydroboration of *O*-acetyl protected cholesterol derivatives. In their model hydroboration of the Δ^5-alkene of cholesteryl-3β-acetate using 20-24 equivalents of borane-THF complex (BH_3-THF), cleavage of the ester was observed even with a range of mildly basic (NaOAc) or neutral pH (phosphate buffer, pH 7.0) oxidiative workups. Despite this, the alkene hydroboration was successful, affording the desired 6α-hydroxyl group. The authors attributed this result to reduction of the ester functionality in competition with the hydroboration of the tri-substituted alkene (hindered and therefore less reactive).

With this in mind, we sought to hydroborate our cholesteryl carbamate **6** which bears four carbamate functional groups which may be sensitive to reduction by BH_3 reagents. First, we repeated the hydroboration of cholesteryl-3β-acetate, with the intention of optimising the reaction conditions, we especially wanted to lower the number of equivalents of BH_3 required, in order that the four less electrophilic carbamates (cf. cyclohexyl esters) would survive and not be reduced to their methylamines (March 1985).

Using BH_3-dimethyl sulfide complex (BH_3-DMS, 10 M solution in DMS) we were able to effect the hydroboration of cholesteryl acetate (Fig. 40, 2.5 eq. BH_3, 25 °C, 4 h, then $NaHCO_3$-H_2O_2 workup, 0 °C, 1 h) to afford initially, mixtures of the desired hydroborated product 6α-hydroxy-5α-cholesteryl-3-acetate **52** and the 3β,6α-diol **53**. After a rigorous literature search, improved yields of acetate **52** following the addition of EtOAc (10 % v/v in CH_2Cl_2) were achieved. The addition of 10 % EtOAc is said to inhibit ester reduction by BH_3, allowing selective reactions of carboxylic acids, alkenes and ketones in the presence of esters (Lane 1976). We used BH_3-DMS due to its favourable stability in air and to heat compared to BH_3-THF (Lane 1975). Also, it is a milder BH_3 reagent, suitable for selective reactions of BH_3

sensitive functional groups as reported by Brown (1982). As well as its convenience of use, it is available at a higher concentration (10 M) than the 1 M BH_3-THF solutions.

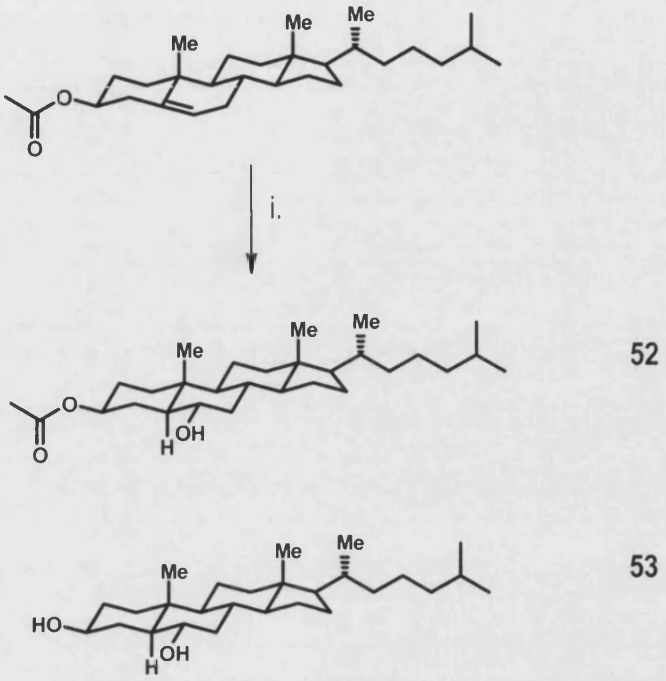


Figure 40. Hydroboration of cholesteryl acetate

i. BH_3-DMS (10 M in DMS), CH_2Cl_2-EtOAc (9:1), 25 °C, 4 h then $NaHCO_3/H_2O_2$, 0 °C

Under these conditions (without the addition of EtOAc), cholesteryl carbamate **6** was hydroborated (Fig. 41) to yield two products, both more polar than the starting material and separable by column chromatography. The major product **54** (65 % yield) was confirmed as the 6-hydroxy regioisomer by the appropriate accurate mass spectrum, and appropriate new signals for 6'-CH resonances in the ^1H NMR (upfield from 5.37 to 3.34 ppm) and ^{13}C NMR (upfield from 122.1 to 69.4 ppm) spectra.

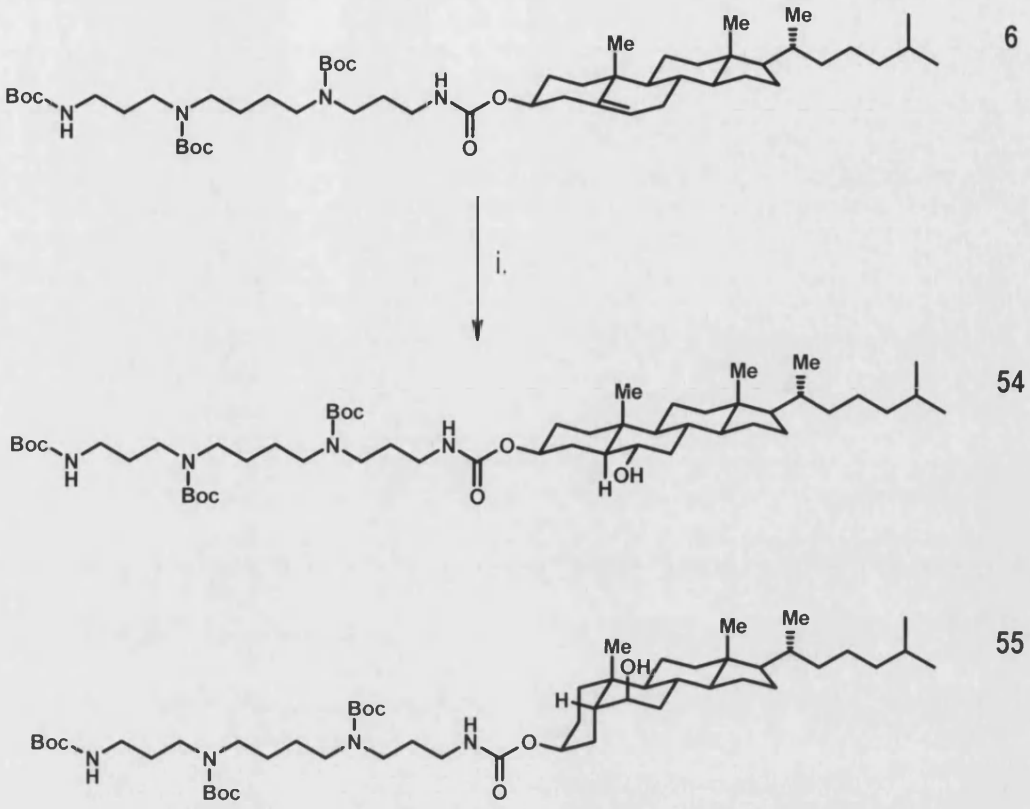


Figure 41. Hydroboration of **6**, to afford the major product **54** and a minor product **55**

i. BH_3-DMS (10 M in DMS), CH_2Cl_2, 25 °C then $NaHCO_3/H_2O_2$, 0 °C

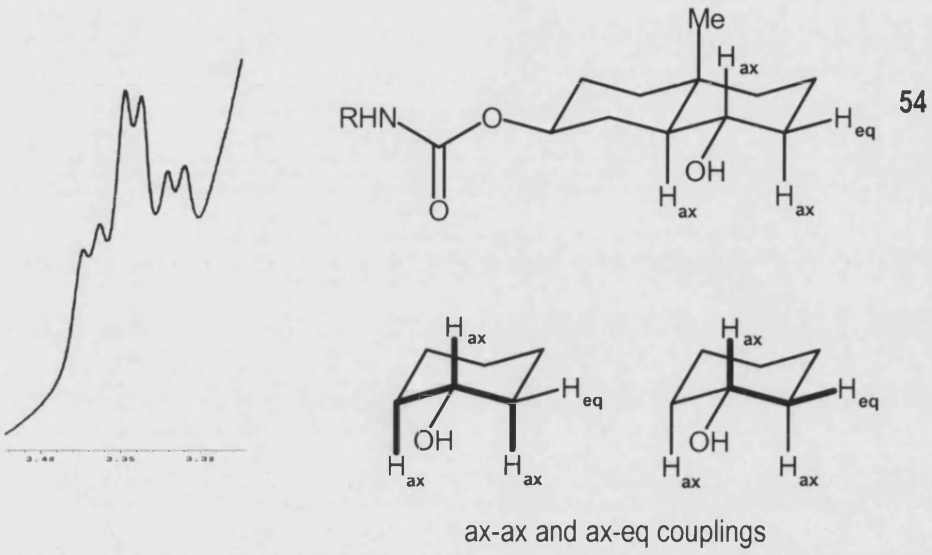
Fig. 42 shows values from the ^{13}C NMR DEPT spectrum (methine carbons only) for product **54** which are in good agreement with our observed values for hydroborated cholesteryl acetate **52** and literature values for 3β,6α-dihydroxy-5α-cholestane **53** (Grover and Stothers 1974).

Assignment	**54**	**52**	3β,6α-dihydroxy-5α-cholestane **53**
3'	73.8	73.6	71.1
5'	51.7	51.7	51.6
6'	69.4	69.5	69.3
8'	34.4	34.4	34.3
9'	53.7	53.7	53.7
14' and 17'	56.1	56.1, 56.2	N/A
20'	35.8	35.8	N/A

Figure 42. ^{13}C NMR assignment for cholesteryl carbamate **54** and ester **52** compared to literature values for 3β,6α-dihydroxy-5α-cholestane **53** (Grover and Stothers 1974). Values expressed in ppm, measured in $CDCl_3$

The minor product **55** was isolated in 3 % yield and found to have the same mass as the major product **54**, with the same fragmentation pattern. Alkene hydroborations are known to be *syn*-additions of H_2O, through the concerted addition of BH_3 (Lane 1974). Based on this and coupling constants measured for 6-H in the ^1H NMR spectrum of **54** (Fig. 43), we assign the major product **54** as the *trans*-AB ring (5α-*H*,6α-hydroxy) product and the minor product **55** as the diastereoisomer (*cis*-AB ring, 5β-*H*,6β-hydroxy). The Karplus relationship predicts $^3J_{HH}$ values according to the dihedral angle. In a 6-membered ring (such as the B-ring of **54**), dihedral angles are fixed, and the Karplus equation predicts the largest coupling constants (typically 9-13 Hz) for antiperiplanar protons (axial-axial coupling) where the dihedral angle is close to 180° (Williams and Fleming 1989). The 6-H of the major product displays two large axial-axial couplings ($^3J_{HH}$ = 10 Hz) and one smaller axial-equatorial coupling ($^3J_{HH}$ = 4 Hz), giving rise to the observed dt at 3.34 ppm (interactions shown bold, Fig. 43). This is consistent with the 6α-hydroxy product (*trans*-AB) and with the findings of Tavares and co-workers who

described the major product of a cholesterol hydroboration as a 6α-hydroxy *trans*-AB product (Tavares *et al* 1993) and also of Izzo *et al* (1998) who also isolated the minor diastereoisomer (10.7:1 6α-6β ratio) of their hydroborated *O*-benzylated cholesterol derivatives.



ax-ax and ax-eq couplings

Figure 43. Portion of the ^{1}H NMR spectrum of alcohol **54**, showing the dt for 6'β-proton and the AB ring geometry of this, the major hydroboration product (only AB-rings shown for clarity)

The high stereoselectivity of this hydroboration for the steroid α-face is consistent with literature reports that cholesterol and other steroid frameworks are hindered by angular methyl groups at positions 10 and 13 (Breslow *et al* 1984, Tavares *et al* 1993, Hanson 1995, Izzo *et al* 1998, Kuhl *et al* 1999).

During this work, Jung and Johnson published (2000) a hydroboration of cholesterol derivatives, again substantiating our own results and those of others (Tavares *et al* 1993) regarding the reactivity and facial selectivity in the hydroboration of steroid alkenes. There is continued and growing interest in compounds with steroid moieties hydroxylated at position 6, particularly in the area of marine natural products. Jung and Johnson (2000) use hydroboration in their total synthesis of xestobergsterol A **56** (Fig. 44), a potent phosphatidyl

inositol phospholipase C inhibitor and mild cytotoxic (against L1210 murine leukaemia cells) obtained from the marine sponge *Xestospongia bergquistia*. Xestobergsterol A **56** and its related compounds possess a *trans*-AB ring junction with a 6α-hydroxyl group (cf. cholesteryl carbamate **54**). In the same year, the structures of three new compounds isolated from the starfish *Goniopecten demonstrans* were published (De Marino *et al* 2000), each a 6α-hydroxylated *trans*-AB ring steroid with anti-biofouling activity against the brown macroalga *Hincksia irregularis*. This is a further example of starfish natural products with this hydroxylation pattern and complements the work of Izzo and co-workers who used a hydroboration reaction to access a *trans*-AB steroid followed by inversion of the major product in order to afford the required 6β-hydroxyl group in the cytostatic compound **57** (Izzo *et al* 1998).

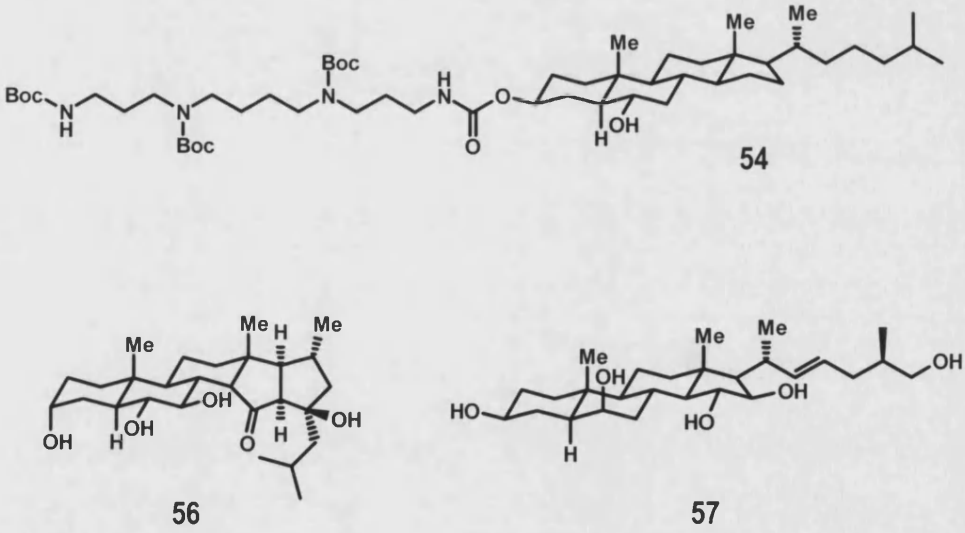


Figure 44. Structures of 6α-hydroxylated polyamine cholesteryl carbamate **54** and two marine natural products, xestobergsterol A **56** and starfish cytostatic **57**

In Chapter 2 we described the synthesis of mono- and polyamine carbamates incorporating the steroid ergosterol as the lipid moiety in novel agents for DNA condensation and non-viral gene delivery. Following the success of our hydroboration of cholesterol as a means of utilising functionality on unsaturated steroids, we searched the literature for ergosterol hydroborations. Ergorsterol **23** acetate (shown in Fig. 45) has a conjugated diene in the B-ring, as well as a remote alkene in the side chain. Hydroboration of ergosterol and its derivatives and other steroidal 5,7-dienes are known in the literature (Nussim *et al* 1964b, Evans *et al* 1975, Goldstein 1996). Here we consider two hydroboration reactions of steroidal dienes (Fig. 45). Nussim *et al* (1964) report boron addition to carbon 6 or 7, followed by hydrolysis of the alkylborane intermediate and rearrangement to afford the Δ^6-alkene **58**. Goldstein (1996) claimed a similar hydroboration of the B-ring of ergosteryl acetate, with the usual oxidation of the alkylborane intermediate to furnish the 6α-hydroxy-7-ene **59**. Goldstein (1996) reported selectivity for hydroboration of the B-ring over the side-chain alkene, with two molar equivalents of borane-DMS.

Selective side-chain hydroxylation of ergosteryl-based cationic lipids, allows fluorescent moieties to be introduced to a steroid side-chain, a probe of SARs in the binding of fluorescent steroidal lipopolyamines to DNA. In order to hydroborate selectively the side-chain of ergosterol, Evans *et al* (1975) protected the B-ring diene with a tricarbonyliron moiety **60**. Using diborane, these workers report the hydroboration of ergosterol, but the regio- and stereoselectivity of this reaction were not investigated. Given these potential problems in hydroboration of the triene ergosterol, we attempted to hydroborate ergosteryl carbamate **33** (Chapter 2) with the dialkylborane 9-borabicyclo[3.3.1]nonane (9-BBN). This reagent has been reported as useful for high regio- and stereoselectivity in hydroborations (Chen 1978, March 1985). Using 9-BBN, we attempted to selectively hydroborate the side chain alkene of ergosteryl carbamate derivatives.

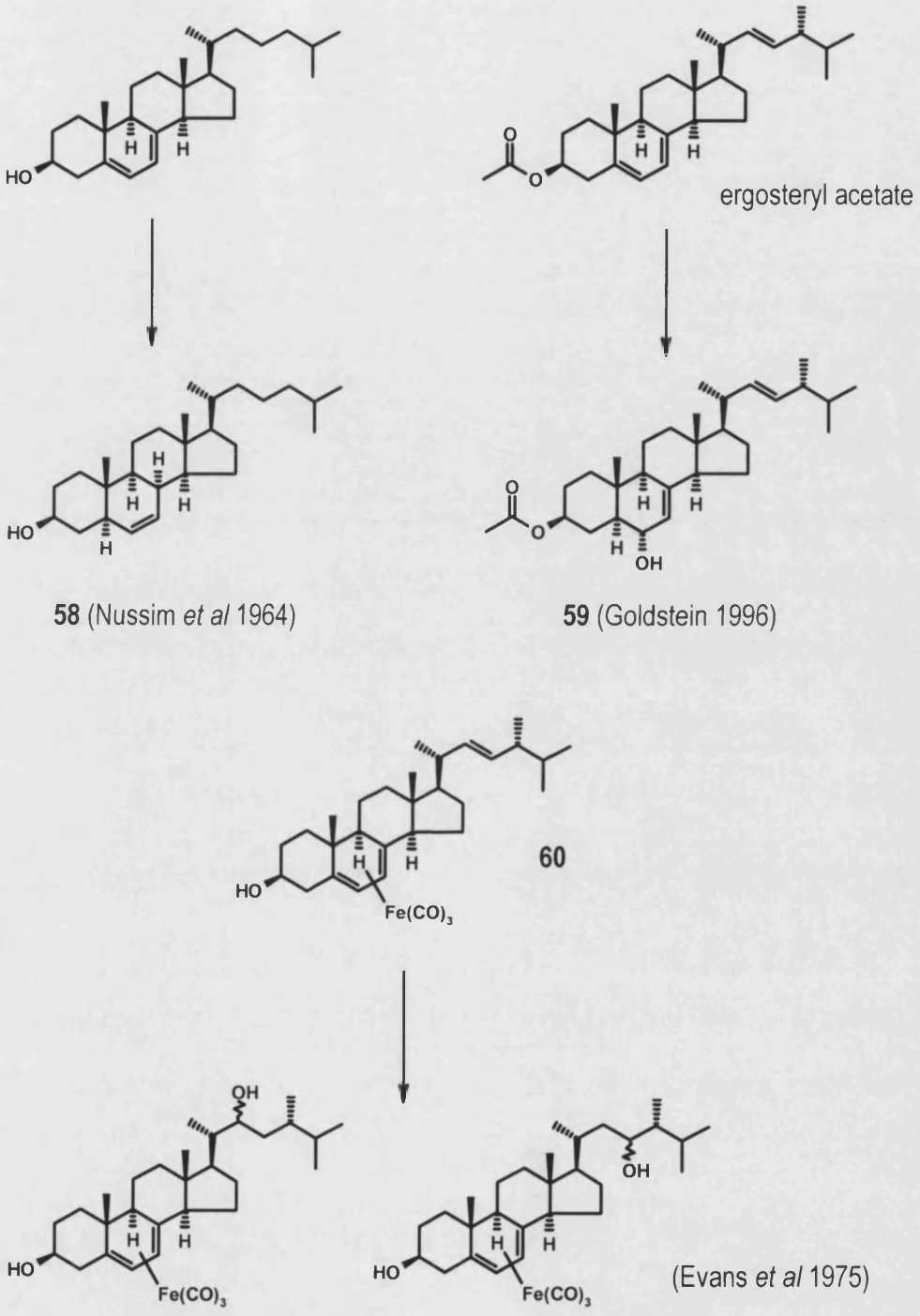


Figure 45. Hydroborations of steroidal B-ring dienes: Nussim *et al* 1964b, Evans *et al* 1975, Goldstein 1996

Treatment of ergosteryl carbamate **33** with 9-BBN (solution in THF) afforded a single more polar spot when monitored by TLC (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), with consumption of starting material **33**. Following treatment of this compound with alkaline H_2O_2, purification of this material over silica gel afforded a product with an identical ^1H NMR spectrum to the starting material. We discount the conclusion that a borane-trialkylamine **33** complex accounted for the more polar component by TLC and identical spectroscopy to starting material **33**. Borane-trialkylamine complexes are known to be useful, commercially available reagents for the hydroboration of alkenes (Lane 1976) and therefore are known to be oxidised by alkaline H_2O_2. We conclude that the initial organoborane intermediate eliminated the alkene starting material **33** upon further manipulation.

Fluorescent lipopolyamine targets based on cholesterol and lithocholic acid

Our hydroboration of polyamine cholesteryl carbamate **6** together with our rapidly accessed lithocholic acid polyamine amide allowed us to design highly fluorescent lipopolyamines based on these *trans*-AB and *cis*-AB steroids. Initially, we conceived targets **61** and **62** (Fig. 46) with spermidine as the polyammonium moiety and incorporating fluorescein, a commonly used fluorophore for confocal microscopy (Boturyn *et al* 1997). Fluorescein is commercially available as the isothiocyanate, making it suitable for covalent labelling of a range of biomolecules to produce highly fluorescent conjugates which may be imaged under a fluorescent microscope. In our designed lipopolyamines, we aimed to conjugate fluorescein isothiocyanate (FITC) as a thiourea to an amino acid chain, to link the fluorophore to the steroid scaffold. The 5-carbon linker is readily available as 5-aminopentanoic acid (5-aminovaleric acid) and we set out to prepare esters of 5-aminopentanoic acid from our hydroxylated steroidal polyamines.

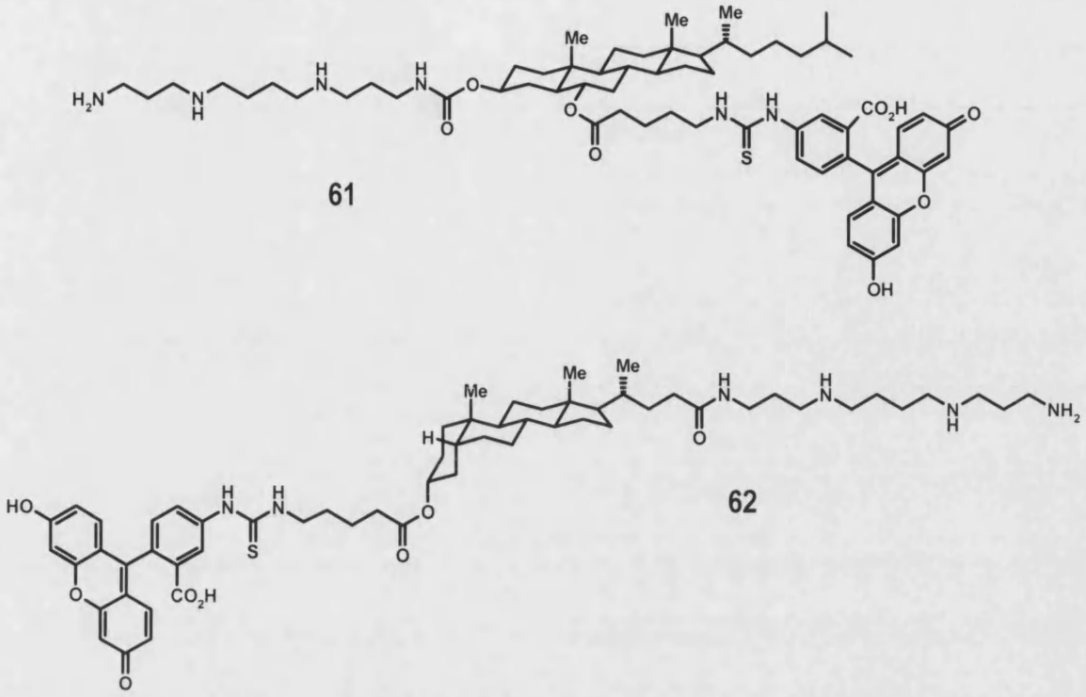


Figure 46. Initial fluorescent lipopolyamine targets **61** and **62**

Initially we aimed to synthesise these targets through a disconnection of the ester thus preparing first the fluorophore-5-aminopentanoate portion then coupling (to make the ester bond) to the poly-Boc protected steroidal polyamine in a convergent route. We prepared three *N*-substituted 5-aminopentanoic acids (Fig. 47) in order that we could couple these, as esters, to the 3α-hydroxy group of our previously described lithocholic acid polyamine amide (Boc-protected to avoid *N*-acylation of the polyamine). Starting from 5-aminopentanoic acid, the amino group was reacted with FITC to afford thiourea **63** (thiocarbonyl, C=S, observed at 182.3 ppm in the ^{13}C NMR spectrum). During these studies, Neves *et al* (2000) reported lissamine rhodamine B (LRB) conjugated plasmid DNA for use in *in vitro* transfection studies tracked by fluorescent confocal microscopy. Lissamine rhodamine B and other examples of rhodamine dyes (Vinayak 1999) are structurally related to fluorescein, but their excitation and emission wavelengths are longer (λ_{ex} = 568 nm, λ_{em} = 583 nm for LRB) relative to fluorescein (λ_{ex} = 494 nm, λ_{em} = 519 nm), making LRB a useful tool for multicolour imaging of two

fluorophores simultaneously. LRB is commercially available as the sulfonyl chloride for conjugation to amines or alcohols. Therefore, as a complement (or alternative) to fluorescein, we synthesised the LRB conjugate, sulfonamide **64**. We also prepared the *N*-carbobenzyloxy carbamate (Cbz or Z protected) of 5-aminopentanoic acid **65** as a model for these short-chain linked fluorophores.

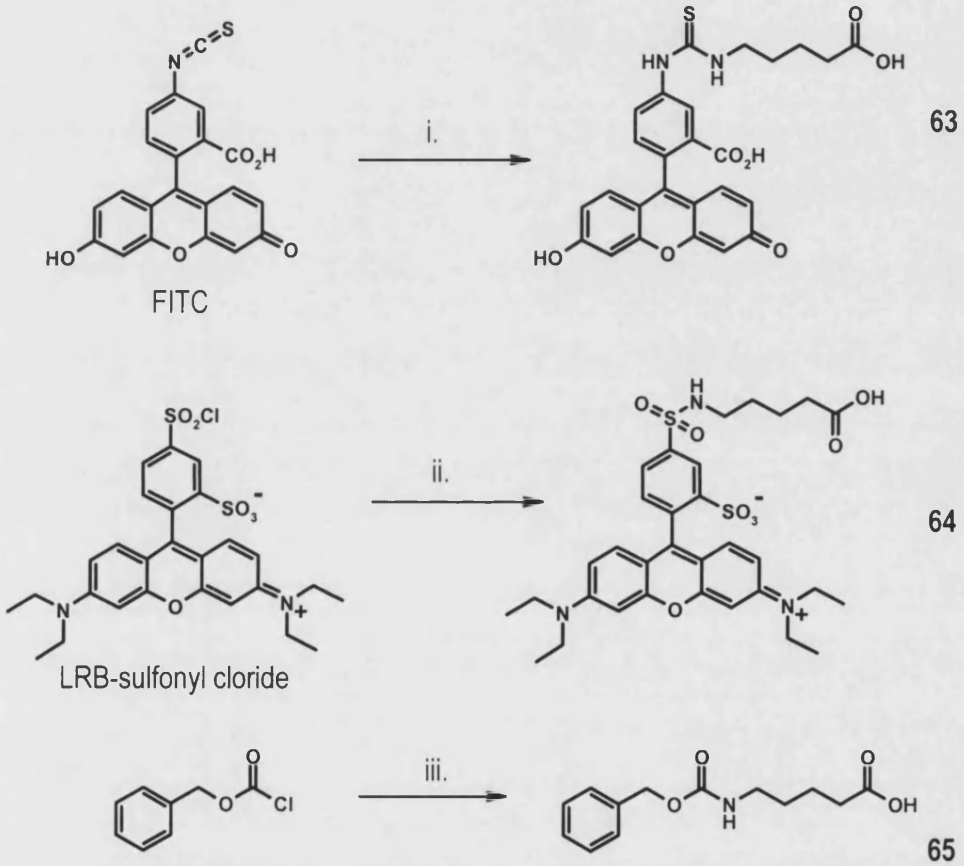


Figure 47. Preparation of chromophore-conjugated 5-aminopentanoic acids **63**, **64** and **65**

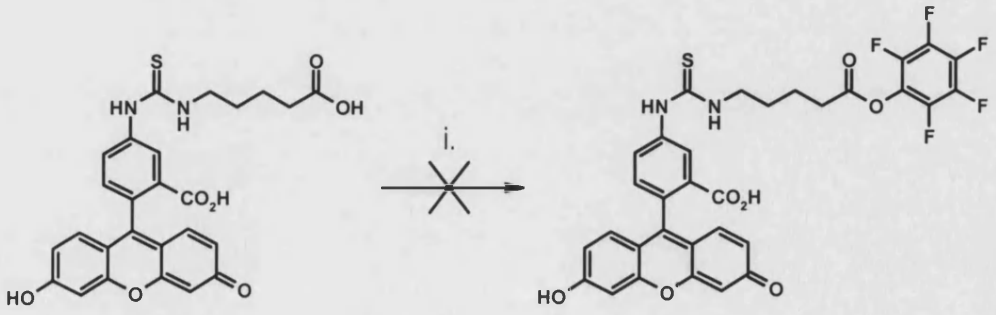
i. 5-aminopentanoic acid, 1 M aq. NaOH, EtOH, 25 °C then pH 3 (2 M aq. HCl);

ii. 5-aminopentanoic acid, 1 M aq. NaOH, acetone, 25 °C then pH 3 (2 M aq. HCl);

iii. 5-aminopentanoic acid, 2 M aq. NaOH, 25 °C then pH 3 (2 M aq. HCl)

We then attempted to esterify these fluorescent pentanoic acids with the previously described lithocholic acid amide **16**. There are several approaches available for the preparation of esters from carboxylic acids and alcohols. Most of these involve activating the acid so that it is electrophilic enough to be effectively attacked by the nucleophilic oxygen of the alcohol. Routes to esters are variations on this transesterification theme, e.g. NHS, HOBt, including the use of acyl halides. Alternatively, Mitsunobu conditions convert the alcohol into a leaving group (cf. conversion into an alkyl halide), for *O*-alkylation, this strategy results in retaining both oxygen atoms of the carboxylic acid in the ester product.

For the preparation of esters of lithocholic acid amides, we first tried to prepare the monopentafluorophenyl (PFP) ester **66** (an "active ester") of FITC conjugate **63** using dicyclohexylcarbodiimide (DCC) coupling (Fig. 48). However, this proved difficult and resulted in inseparable mixtures of starting material and more and less polar components. The FITC and LRB conjugates were poorly soluble in appropriate organic solvents for these reactions, and the FITC conjugate contains a benzoic acid which may have prevented the desired reaction. Also, we used the significantly less expensive mixture of FITC regioisomers (the isothiocyanate at position 5 – "isomer I" and position 6 – "isomer II" 9:1) which were separable on silica.



63 **66**

Figure 48. Attempted selective pentafluorophenyl esterification of FITC conjugate **63**.

i. Pentafluorophenol, EtOAc, DCC, 25 °C

In order to conserve the fluorescent conjugates, we pursued this route by next making the acid chloride of Z-protected 5-aminopentanoic acid **65** using oxalyl chloride in toluene. After quantitative consumption of the starting material (monitored by TLC) the resulting solution was evaporated to dryness and the resulting crude mixture was found to be 86 % acid chloride **65a** by ^{13}C NMR (acid chloride carbonyl at 170.7 ppm). However, attempts to esterify this mixture by heating (50 °C) and later refluxing with alcohol **16** in anhydrous pyridine failed to yield the desired product. Instead, we obtained mixtures of starting alcohol **16** and acid **65**, possibly due to the low nucleophilicity of the secondary alcohol and also because of hydrolysis of the acid chloride as the solvent became wet on extended reaction times.

Synthesis of Fmoc-protected aminopentanoate esters and their rapid deprotection for the generation of fluorescent lipopolyamine libraries

The synthesis of fluorescent lipopolyamines via esterification of the 3α- and 6α-hydroxyl functional groups of synthetic poly-Boc protected lipopolyamines was achieved by a linear route. Initial attempts, which focused on introducing convergence to the total synthesis by coupling activated fluorophores to 5-aminopropanoic acid, prior to esterification with the steroid core, were abandoned due to solubility problems of these fluorescent conjugates and the current high cost associated with obtaining practical quantities of FITC, LRB-sulfonyl chloride, and other activated fluorophores, some available only in single milligram quantities.

To aid these synthetic studies of fluorescent lipopolyamines, a model lithocholic acid amide **67** was synthesised from the acid and *n*-propylamine. Unfortunately, this product, prepared from the DCC/HOBt coupling reaction, was insoluble in the range of deuterated solvents available, therefore we were unable to characterise the product by NMR, which we hoped would be of use in subsequent NMR assignments.

At the same time, we investigated the possibility of using a 9-fluorenylmethoxycarbonyl (Fmoc, Carpino and Han 1970, Atherton *et al* 1978) protecting group to mask the primary amine of our designed lipopolyamine steroid esters of 5-aminopentanoic acid. Fmoc is a common nitrogen protecting group (for a recent review of nitrogen protecting groups, see: Theodoridis 2000), especially known in the area of automated solid phase synthesis (Li and Chou 2000) and peptide synthesis (Ueki and Ameniya 1987), where its deprotection may be rapidly monitored by fluorescence. Fmoc carbamates may be deprotected by bases, particularly secondary amines (e.g. piperidine), and Fmoc groups are resistant to strong acid. These properties are in contrast to the widely used Boc protecting group and the two protecting groups may be orthogonally manipulated (Greene and Wuts 1991). In addition, Fmoc carbamates are inert to hydrogenation conditions, making Fmoc a useful alternative to the Z carbamate, should a synthetic sequence require hydrogenation (Li *et al* 1993a and 1993b).

Therefore, we conceived a new linear route to our designed fluorescent lipopolyamines based on selective deprotection of Fmoc carbamates in order to introduce fluorescent labels. In Fig. 49 we outline this route, which we investigated starting with the model amide of lithocholic acid **67** and Fmoc-protected 5-aminopentanoic acid **68**.

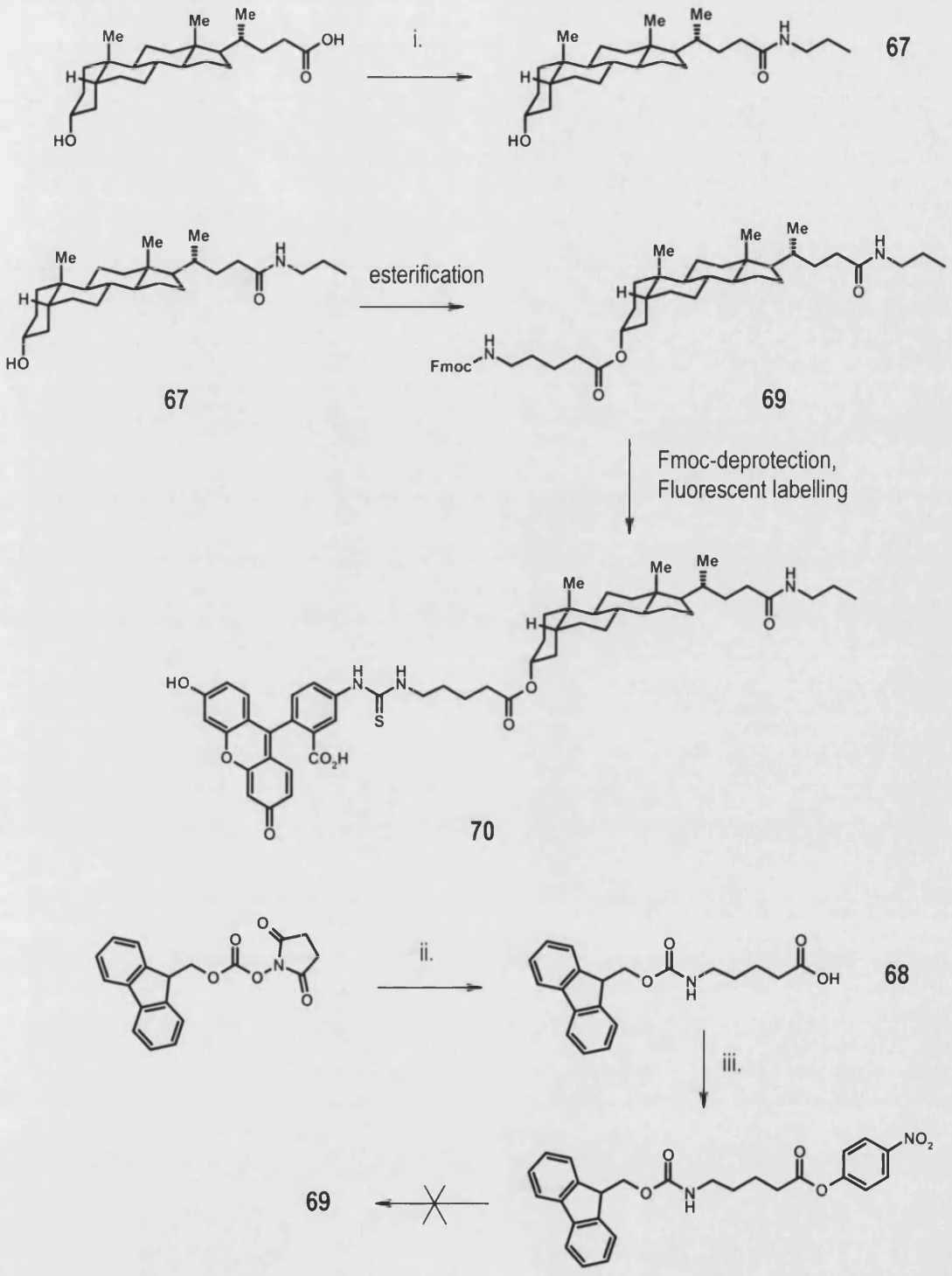


Figure 49. Proposed synthesis of fluorescent lithocholic acid amides via Fmoc-protected amino esters.

Synthesis of Fmoc-protected 5-aminopentanoic acid **68**

i. 1-propylamine, CH_2Cl_2, DCC, HOBt, 25 °C, 3 h; ii. 5-aminopentanoic acid, $NaHCO_3$, dioxane-water (3:2), 25 °C then pH 3 (2 M aq. HCl); iii. 4-nitrophenol, DCC, EtOAc, 25 °C, 16 h

The Fmoc-protecting group may be introduced with a variety of activated reagents. We used the *N*-hydroxysuccinimide ester (NHS) according to the procedures of Beyermann *et al* (1990). The reaction with 5-aminopentanoic acid proceeded cleanly in dioxane-water (ratio 3:2) to afford the desired product **68** (82 % yield). Although the esterification of this Fmoc-protected amino acid is analogous to the efforts previously described, the reaction in rigorously dried CH_2Cl_2 with pre-activation with DCC/DMAP succeeded after attempts using the acid chloride in pyridine and the *para*-nitrophenyl (PNP) ester **71** both failed. This less polar product **69** was confirmed as the ester by ^{1}H and ^{13}C NMR (from which we inferred the identity of amide **67**), in which we observed three quaternary carbon resonances at 156.2, 172.7, 173.2 which we identify as the carbonyls of carbamate, ester and amide. We can assign with confidence the carbamate to 156.2 ppm. Typically, secondary amides of lithocholic acid (e.g. amide **16**) appear at around 174.0 ppm. However, without the data for the starting material, propylamide **67**, we do not over interpret the data here.

We required a one-pot reaction for the Fmoc deprotection step, followed by fluorescent labelling *in situ*. This approach avoids the need for purification of the deprotected amine intermediate. Piperidine is a commonly used base in the deprotection of Fmoc groups, but as a secondary amine it is nucleophilic and reactive to electrophiles such as isothiocyanates. We predicted an excess of piperidine would compete with the deprotected primary amine for reaction with FITC. Therefore, this reagent was unsuitable for our requirements, without rigorous extraction. In solid phase organic synthesis, piperidine is removed by extensive washing of the resin in the rinse cycle. An alternative to piperidine is tetra-*n*-butylammonium fluoride (TBAF, Greene and Wuts 1991), which despite being a well-known reagent for deprotecting silicon based protecting groups, has so far found only limited application in the literature for cleaving Fmoc carbamates (Ueki and Ameniya 1987, Wilson *et al* 1999).

Using an excess of TBAF (1 M solution in THF), the deprotection of 50 mg of **69** was complete within two minutes, as monitored by TLC, yielding a polar spot which stained with ninhydrin. Methylidenefluorene, the elimination product of an Fmoc deprotection, was also detected as a UV absorbing non-polar component. This was followed by the addition of an equimolar quantity of fluorescein isothiocyanate, isomer I (Molecular Probes, Oregon), giving a visible (green), less polar compound by TLC which stained with PMA and was fluorescent (green) when visualised at 366 nm. Despite the apparent success of the reaction, attempts to purify the mixture were largely unsuccessful. However, FAB accurate mass data were acquired, displaying the appropriate elemental composition for the desired product **70** (FAB-MS *m/z* 906 (MH^+), $C_{53}H_{67}N_3O_8S$ requires 905; FAB-HRMS $C_{53}H_{68}N_3O_8S$ requires 906.4727 (MH^+), found 906.4762).

Interpreting this result as a proof-of-principle, we began the synthesis of a range of fluorescent lipopolyamines based on spermidine and spermine polyammonium moieties. In using the Fmoc deprotection chemistry, we have devised a synthetic route that enabled the introduction of a variety of fluorophores. Our new immediate targets were therefore lipopolyamine Fmoc esters incorporating lithocholic acid (Fig. 50 and 51) and cholesterol (Fig. 52).

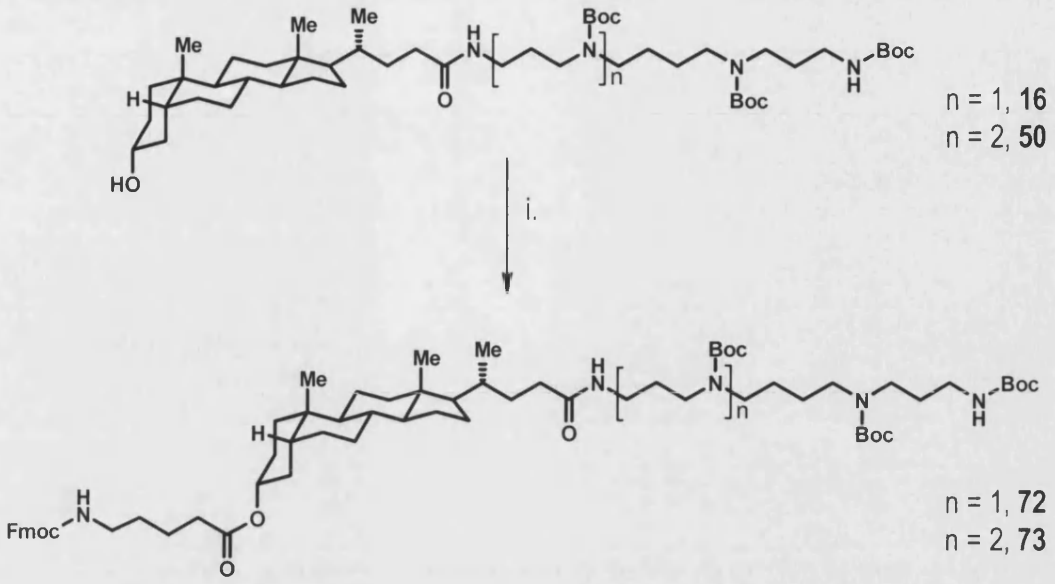


Figure 50. Precursors to fluorescent polyamine lithocholic acid amides: Fmoc protected esters **72** and **73**.

i. **68**, DCC, HOBt, CH_2Cl_2, 25 °C

Starting from poly-Boc protected 3.4.3-Litho **16** and 3.4.3.3-Litho **50** (previously described in Chapter 2), we prepared esters of Fmoc-protected 5-aminopentanoic acid **68** using DCC/HOBt coupling as with the model **69**. These reactions (Fig. 50) proceeded smoothly in anhydrous CH_2Cl_2 and, after column chromatography, afforded protected spermidine mimic **72** and spermine mimic **73**, typical yields were 40-80 %, the highest yields were obtained when the reaction was stirred for two days.

Satisfactory ^1H and ^{13}C NMR data were obtained for these esters (3'β-H shifted downfield from 3.6 to 4.7 ppm in compound **73**, typical of a C<u>H</u>-OH to C<u>H</u>-OCO transformation, Williams and Fleming 1989). Appropriate FAB mass spectral data were obtained, although we were unable to collect an accurate mass spectrum for compound **73** (the low resolution mass was correct). Requiring a M+1 m/z of 1339, this coincided with m/z 1339 of the polyethylene glycol monomethyl ether (PEG-MME) reference used for accurate mass measurements (University of Bath Mass Spectrometry service). Upon advice from the EPSRC National Mass

Spectrometry Service (University of Swansea) we sought the use of CsI clusters as references for accurate mass measurement of compound **73**. We were further advised by the EPSRC MS Service that, in this case, accurate mass data are only as reliable as low resolution data for elemental formula determination at M+1 over 1000.

In order to demonstrate the poly-Boc deprotection of such compounds and the stability of the ester linkage towards the strongly acidic conditions (TFA) used in such deprotections, we treated compound **72** with TFA (1:9 v/v solution in CH_2Cl_2) for 3 h, then purification of 10 mg of this crude material by semi-preparative HPLC (isocratic mobile phase: 0.1 % aq. TFA-MeCN 20:80 % v/v) afforded the spermidine mimic **74** (Fig. 51).

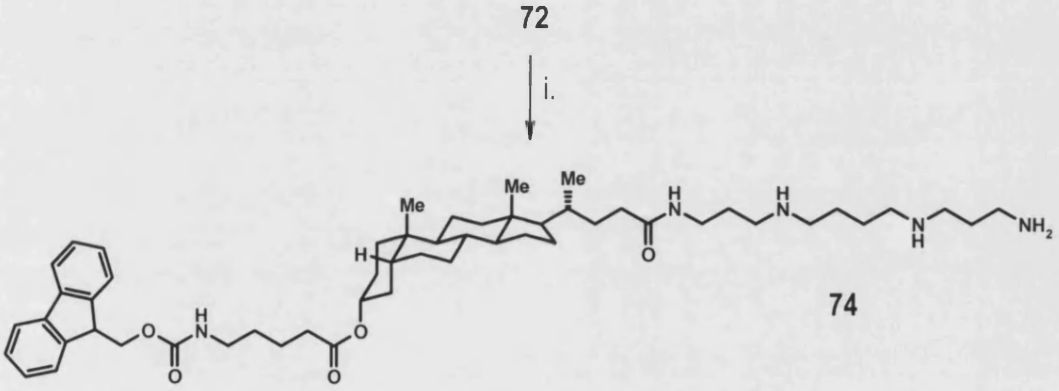


Figure 51. Synthesis of Fmoc-labelled lithocholic acid amide **74**.

i. TFA-CH_2Cl_2 (1:9), 25 °C, Semi-Prep RP-HPLC

Accurate FAB$^+$ MS data for this compound indicated the desired fully deprotected compound with the ester and Fmoc carbamate intact ($C_{54}H_{84}N_5O_5$ requires 882.6472 (MH^+), found 882.6444). Polyamine conjugate **74** is a fluorescent lipopolyamine, although the excitation wavelength of the Fmoc fluorophore is low in the UV region (λ_{ex}: 265 nm), out of the range of most modern confocal microscopes, making it unsuitable for sensitive cell imaging (Johnson 1998). The fluorescence of our Fmoc conjugate **74** was determined to be λ_{max} (Ex): 265 nm, λ_{max} (Em): 320 nm.

Next we prepared the analogous cholesterol derivatives **76** and **77**, starting with Boc-protected polyamine cholesteryl carbamates **6** and **48** respectively (Fig. 52).

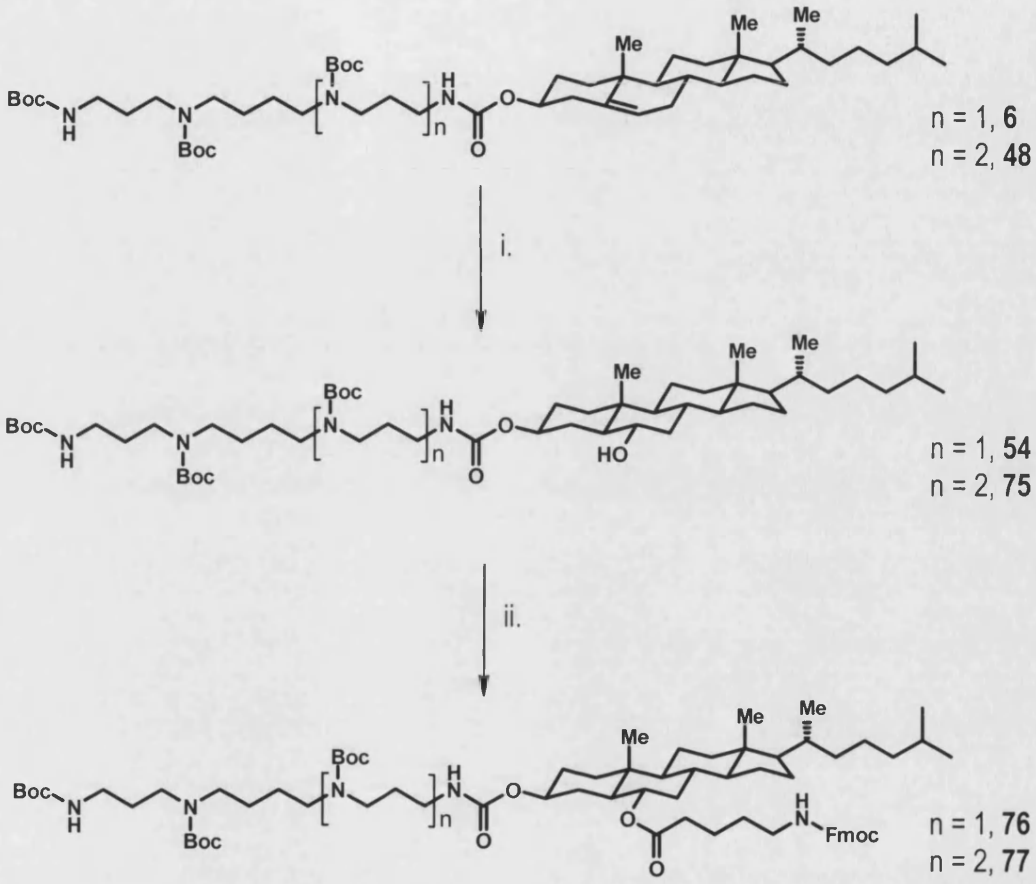


Figure 52. Synthesis of precursors to fluorescent polyamine cholesteryl carbamates: Fmoc protected esters **76** and **77**.

i. BH_3-DMS (10 M in DMS), CH_2Cl_2, 25 °C then $NaHCO_3/H_2O_2$, 0 °C; ii. **68**, DCC, HOBt, CH_2Cl_2, 25 °C

Spermidine mimic tri-Boc-3.4.3-Chol **6** was hydroborated as previously described to afford alcohol **54** and spermine mimic tetra-Boc-3.4.3.3-Chol **48** was reacted according to the same procedure, affording alcohol **75**. These 6α-hydroxyl groups were then acylated in an analogous manner to the lithocholates affording esters **76** and **77** respectively. Upon *O*-acylation, the dt (^{1}H NMR) associated with the methine proton at 6-C shifts downfield from 3.38

ppm in alcohol **75** to 4.68 for the desired ester **77**. In the ^{13}C spectra the methine carbon at position 6 is deshielded by the ester, downfield from 69.6 to 72.7 ppm in ester **77**. This was accompanied by the shielding of 5-C, upfield from 52.0 to 49.2 ppm. Analogous shifts were observed for the esterification of **54** (^{1}H NMR: dt 3.34, ^{13}C: 69.4) to **76** (^{1}H NMR: dt 4.69, ^{13}C: 72.3). These spectral effects upon *O*-acylation are in agreement with NMR shift additivity rules (Williams and Fleming 1989).

Choice of fluorophore

Having designed precursors to fluorescent-labelled steroidal polyamine derivatives we had established a route by which we can introduce a range of fluorophores that will find ready application in the imaging of intracellular transfection events. One of many, or a combination of considerations may influence the choice of fluorophore label for confocal microscopy work. These include the spectral properties of the fluorophore (for example excitation/emission wavelength, pH dependent fluorescence, photobleaching, quantum yield). Also, the availability of fluorophores that are easily incorporated into a labelling synthesis might be an issue (activated, reactive fluorophores, for example isothiocyanates are available; some are marketed in the form of labelling "kits").

Fig. 53 shows of some the various structures that contribute to the wealth of fluorophores available for intracellular imaging by fluorescence microscopy. These examples are all appropriate to our requirements in that, in their activated forms, they may be used to label our steroidal lipopolyamines **72**, **73**, **76** or **77**, following an Fmoc deprotection with a fluoride reagent such as TBAF.

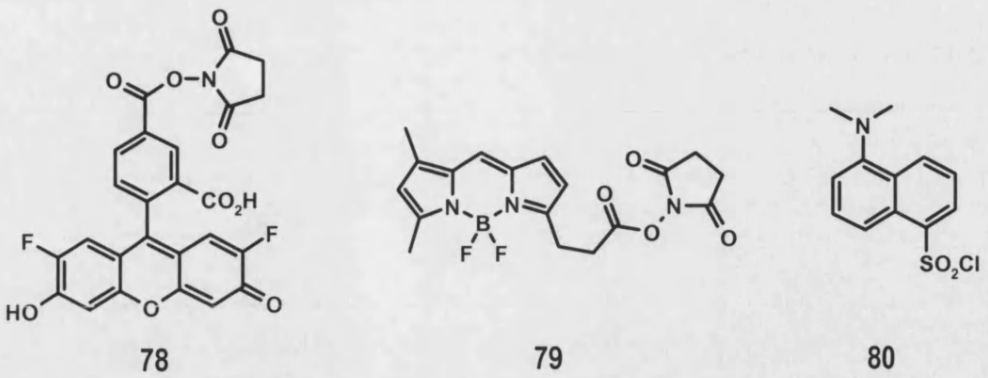


Figure 53. Structures of some commercially available fluorophores: Oregon Green 488 NSE **78**, BODIPY-FL NSE **79** and dansyl chloride **80**

Synthesis of fluorescent alkylspermine cholesteryl carbamates

As discussed in Chapter 1, rhodamine fluorophores (such as lissamine rhodamine B, LRB, see our earlier synthetic work in this chapter) have been incorporated into RPR-121653 **81** (Fig. 54). This fluorescent thiourea was synthesised from the commercially available tetramethylrhodamine B isothiocyanate (Byk *et al* 1998).

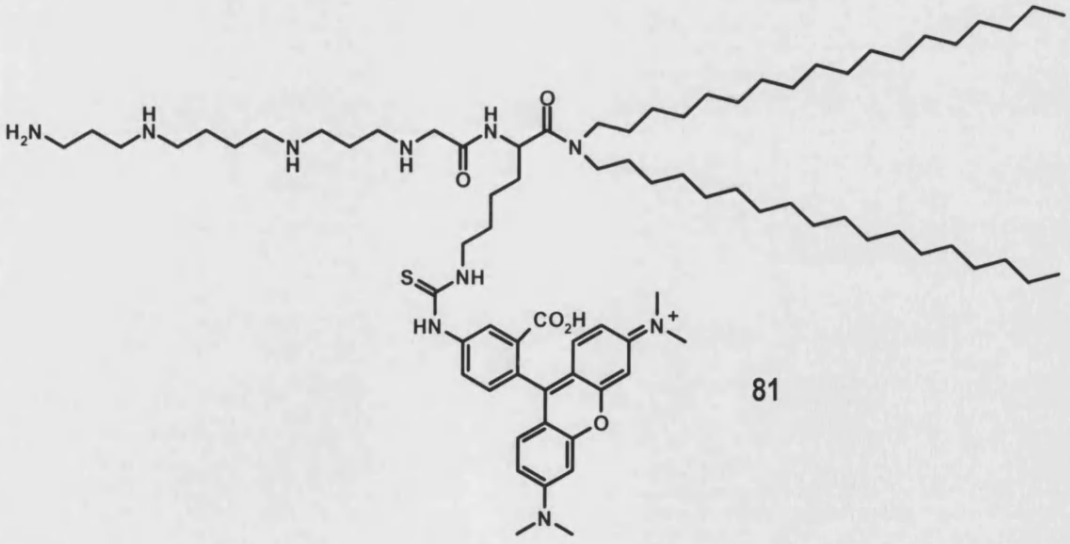


Figure 54. The fluorescent lipopolyamine RPR-121653 **81**

Complementary to the red rhodamine-type fluorophores is Oregon Green 488 (2',7'-difluorofluorescein). This and other green fluorophores with a fluorescein-like fluorescence profile have spectra that do not overlap with rhodamines and therefore are suitable for use together in dual-colour imaging experiments. The "488" refers to the wavelength, in nm, of the spectral line of the argon ion laser, used in a fluorescence microscope. The absorption maximum (498 nm) of Oregon Green 488 closely matches this emission line. A range of these dyes is available to match other laser emission lines, for example Oregon Green 514. Oregon Green 488 is available as an alternative to fluorescein, offering improved photostability (illumination of biological samples may cause irreversible destruction of the fluorophore – "photobleaching"). The phenolic pK_a of Oregon Green 488 is 4.7, cf. 6.4 for fluorescein. When the phenol is protonated the fluorescence intensity is diminished (Sun *et al* 1997). Therefore, Oregon Green may be used to image acidic organelles (such as lysosomes, pH 5.5), useful in non-viral gene delivery systems where endosome escape and lysosomal degradation are barriers to efficient transfection (Sun *et al* 1997).

These properties of Oregon Green 488 were persuasive in our choice of fluorophore, together with our previous experience in handling fluoresceins (see model conjugate **70** described previously). Also, its precedented use in multicolour imaging of gene delivery systems (Godbey *et al* 1999b) with rhodamine-labelled DNA (Vinayak 1999, Neves *et al* 2000, Yoo and Juliano 2000) encouraged us to design Oregon Green 488 labelled polyamine cholesteryl carbamate **82** (Fig. 55).

Starting with Fmoc-protected **77**, treatment with TBAF in THF and immediate addition of Oregon Green 488 NSE gave a complicated mixture of spots when monitored by TLC. Working on a small scale (this fluorophore was available in 5 mg units for £120), the separation of this mixture was not attempted. It appeared the presence of TBAF was causing components to run faster with streaking. A careful literature search for examples of solution

phase Fmoc-deprotections followed by amine derivatisation *in situ*, yielded only three reports of KF used to cleave Fmoc carbamates in one-pot procedures in the presence of acylating agents (Li *et al* 1993a and 1993b, Li and Chou 2000). Using these procedures, Fmoc protected **77** was treated with KF in DMF and TEA, then Oregon Green 488 NSE added. On this occasion, clean TLC allowed us to monitor the deprotection through the appearance of the methylidenefluorene by-product and the absence of the deprotected amine (by comparison with a "blank" deprotection, that is the same reaction, without the fluorophore present). These TLC were performed in basic (NH_3 containing) mobile phases, but attempts to resolve the fluorescent baseline spots with other mobile phases failed. However, we were able to infer the presence of a poly-Boc protected fluorescent conjugate by staining the fluorescent spots with PMA.

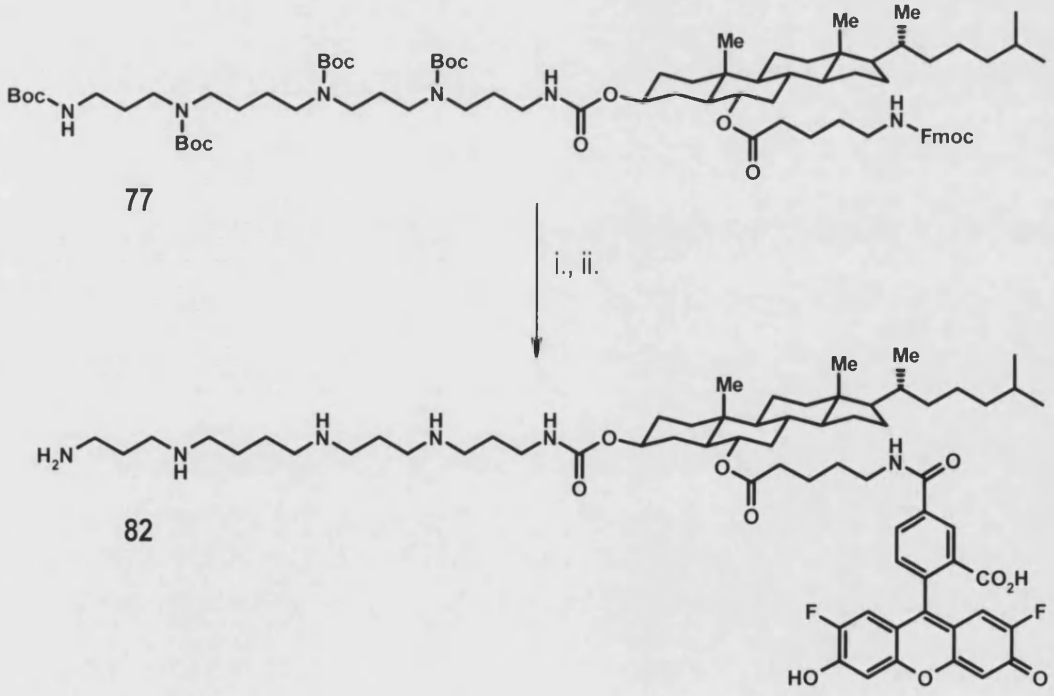
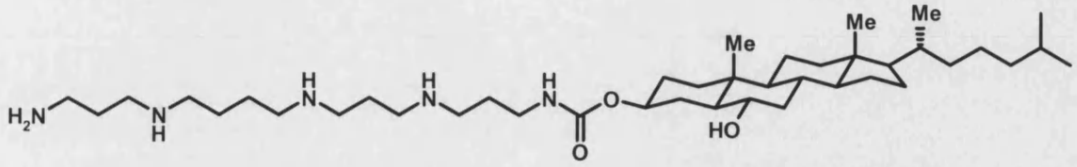


Figure 55. Synthesis of Oregon Green labelled alkyl-spermine cholesteryl carbamate **82**

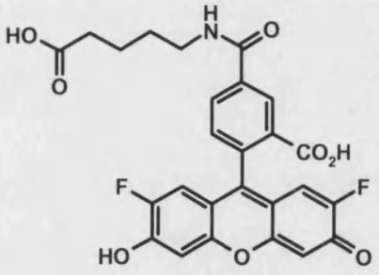
i. KF, Oregon Green 488 NSE, TEA, DMF, 25 °C; ii. TFA-CH_2Cl_2 (1:9), 25 °C, Semi-Prep RP-HPLC

Without purification, this mixture was treated with TFA in order to effect the poly-Boc deprotection, then the reaction was stopped after 3 h and the mixture purified by semi-preparative RP-HPLC (t_R 4.5 min, λ = 488 nm, MeCN-0.01 % aq. TFA 9:1 v/v). The collected peak corresponded to an intense green product (in solution) which was fluorescent green under UV light (366 nm) and stained red with ninhydrin, indicating the polyamine moiety. The normal absorption and fluorescence spectroscopy were also appropriate for product **82** (absorption λ_{max} (H_2O): 489 nm, fluorescence λ_{max} (Ex): 490 nm, λ_{max} (Em): 528 nm). However, the mass spectrum of this compound showed that at some point between purification and FAB sample ionisation, the ester in **82** had degraded to give the alcohol **83** and the Oregon Green conjugate of 5-aminopentanoic acid **84**. Alcohol **83** had further reacted with TFA to form the trifluoroacetyl ester **85** or an isomeric trifluoroacetamide (Fig. 56). Further evidence for the success of the reaction and subsequent purification, is that we predict acid **84** will have a longer column retention time than the peak collected, based on the model FITC conjugate **63** (t_R 12.9 min, λ = 495 nm, MeCN-0.1 % aq. TFA 8:2 v/v). We conclude we have satisfactorily purified fluorescent lipopolyamine conjugate, the desired product **82** and not collected an unresolved mixture of alcohol **83** and acid **84**, which would display similar fluorescence and ninhydrin staining as polyamine ester **82**.

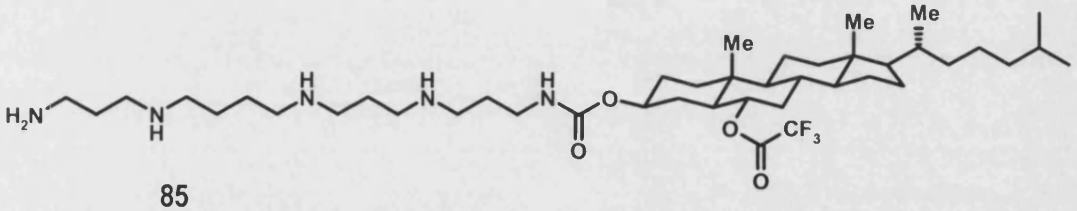
As discussed in Chapter 1, during our studies in non-viral gene delivery, Oregon Green 488 labelled polyethylenimine (PEI) has been used by Godbey *et al* (1999b and 1999c) in transfection experiments, though detailed imaging studies of endosome related events were not reported. However, Oregon Green labelled PEI was observed to translocate to the nucleus in the absence of DNA (Godbey *et al* 1999b). In a similar study, labelling with Oregon Green increased the transfection activity of PAMAM dendrimers (Yoo and Juliano 2000) although any specific translocating role played by Oregon Green remains unreported. However, Oregon Green conjugates remain of interest in this area of non-viral gene delivery.



83
$C_{41}H_{79}N_5O_3$ requires 689



84
$C_{26}H_{29}NO_8F_2$ requires 511



85
$C_{43}H_{78}N_5O_4F_3$ requires 785

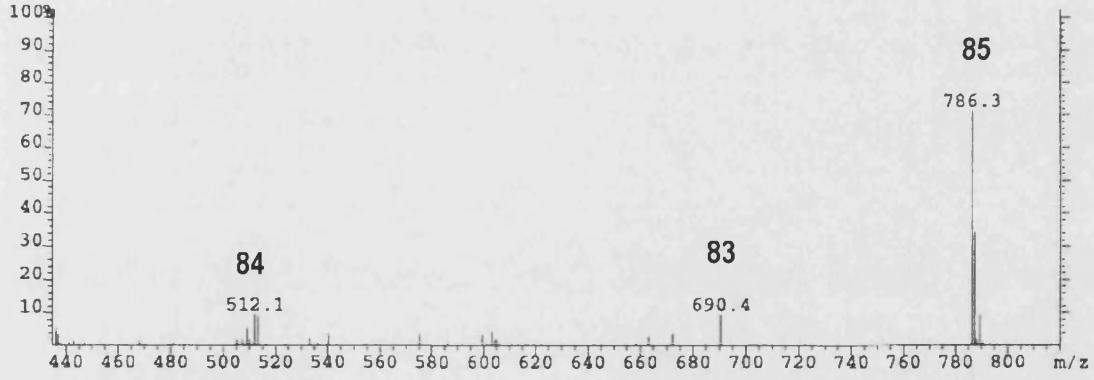


Figure 56. FAB$^+$ mass spectral evidence for the degradation of **82**

We also investigated the dansyl labelling of lipopolyamines, using the reagent dansyl chloride **80** (Fig. 53). Dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) is a commonly used reagent for the fluorescent labelling of amino acids and peptides. Dansyl

chloride is non-fluorescent, but it reacts with nucleophilic amines, affording fluorescent sulfonamides (dansyl amides). Here we report the coupling of this reagent with N^1,N^2,N^3-tri-Boc spermine **5** (Fig. 57), and later with a polyamine cholesteryl carbamate (Fig. 58). Fig. 57 illustrates the single, regiochemically-controlled labelling of protected spermine **5** with the dansyl fluorophore, followed by the selective removal of the Boc protecting groups.

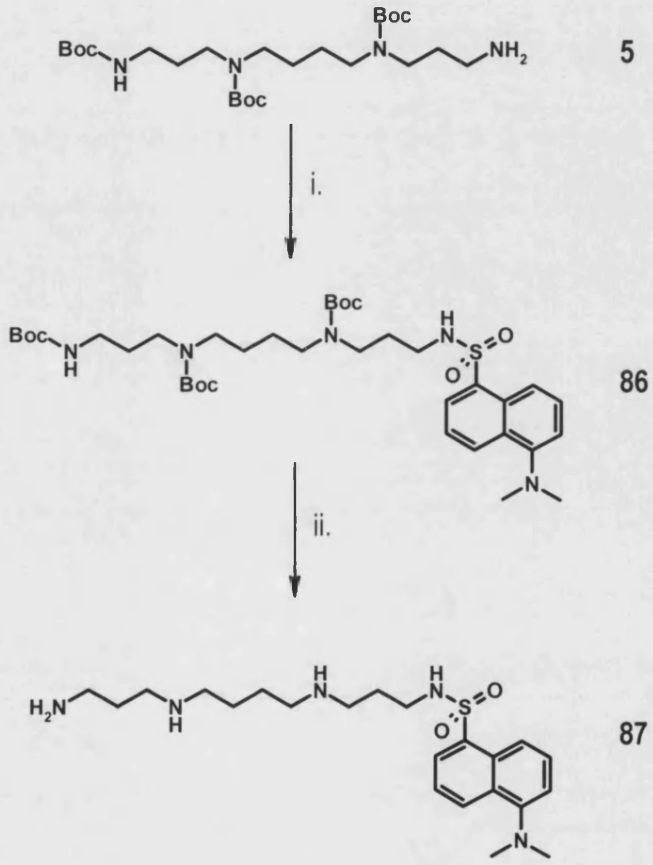


Figure 57. Synthesis of dansyl labelled spermine **87**

i. **80**, CH_2Cl_2, TEA, 25 °C, 5 h; ii. TFA-CH_2Cl_2 (1:9), 25 °C, 3 h

N^1,N^4,N^9-Tri-Boc spermine **5** was reacted with dansyl chloride affording a less polar product **86** by TLC (R_f 0.82 compared to 0.20 for **5**, CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). The product **86**, which had similar chromatography to dansyl chloride **80** was distinguishable from the yellow non-fluorescent starting material by a blue-green fluorescence when visualised under UV light (366 nm). Compound **86** was deprotected with TFA-CH_2Cl_2 (1:9) to afford the monodansylated spermine conjugate **87** (FAB-HRMS $C_{22}H_{38}N_5O_2S$ requires 436.2746 (MH^+), found 436.2734).

Dansylated spermine conjugates are of interest in the detection and analysis of polyamines which, in possessing poor spectroscopic properties, may be difficult to detect either sensitively or quantitatively (Page *et al* 1998, Carrington *et al* 1999). Cationic dansyl amide derivatives are known to interact with duplex DNA (Wang and Schneider 1998) and the dansyl fluorophore is sensitive to lipid environments (displaying enhanced fluorescence in non-polar solvents or certain cellular environments, Wang and Schneider 1998). These are potentially useful properties for the study of DNA-lipopolyamine particle formation and intracellular transfection processes. Therefore, we included dansyl in our designed fluorescent lipopolyamine probes. The synthesis of dansylated **87** provided us with a model route to our proposed lipopolyamine-dansyl conjugates. With this in hand, we followed our fluoride Fmoc deprotection procedure to introduce a dansyl label to cholesteryl carbamate **77** (Fig. 58).

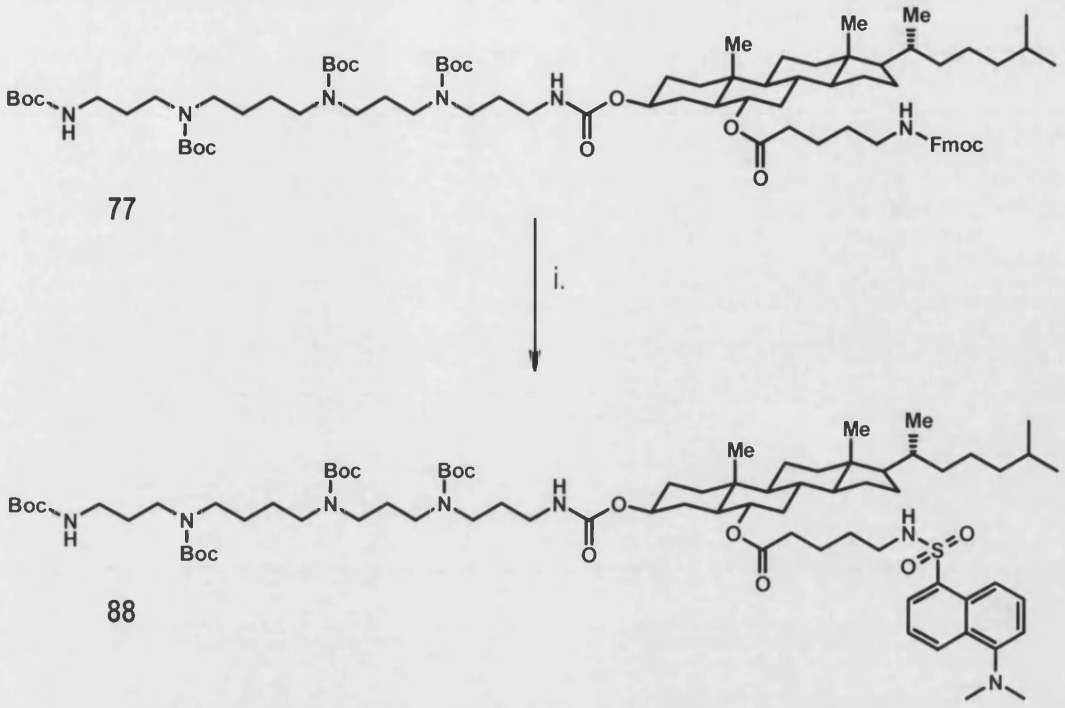


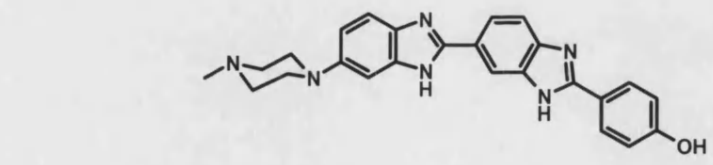
Figure 58. Synthesis of dansyl labelled poly-Boc protected cholesteryl carbamate **88**

i. KF, **80**, TEA, DMF, 25 °C, 3 h

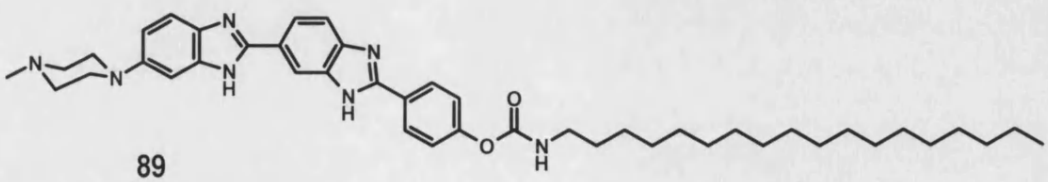
Once again, KF was found to be a convenient reagent for the Fmoc deprotection, and dansyl labelling followed *in situ*, the reactions were monitored by TLC (EtOAc). A new, non-polar spot (R_f 0.93) displaying the distinctive blue-green fluorescence (excitation at 366 nm), stained with PMA, indicating fluorophore and Boc-protected polyamine present in this component, was assumed to be dansyl conjugate **88**. Unreacted dansyl chloride, possessing similar (although resolved) chromatography, was distinguished as a visible bright yellow, non-fluorescent spot which did not react with PMA. Although the product **88** and dansyl chloride were separable by chromatography, it was not practical on this single milligram scale. Instead, the product **88** was washed into ether, affording a rapid separation from polar materials (e.g. the excess of KF, triethylammonium chloride). The product **88** displayed the desired accurate mass spectrum (FAB-HRMS $C_{78}H_{132}N_7O_{14}S$ requires 1422.9553 (MH^+), found 1422.9454). This Boc-protected product **88** was treated with TFA-CH_2Cl_2 (1:9 v/v). A more polar, baseline

spot, monitored by TLC (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v), was formed though no attempt was made to purify the lipopolyamine-dansyl conjugate due to time constraints.

The non-viral gene delivery vectors described so far are cationic lipids, and interact with DNA through electrostatic interactions between the cationic moiety and the anionic polyphosphate backbone of DNA. These compounds possess weak UV chromophores and require the introduction of a fluorescent label in order to be visualised by spectroscopic techniques. During our synthetic studies of fluorescent lipopolyamines, a novel gene delivery system based on non-electrostatic groove-binding has been designed and synthesised (Soto *et al* 2000). Carbamate **89** (Fig. 59) is a single C18 lipid chain conjugated to Hoechst 33258. Hoechst 33258 is known to bind in the minor groove of double-stranded DNA (Moon *et al* 1995). Although conjugate **89** contains a basic piperazine moiety which may be protonated at pH 7.4, the authors report that binding of conjugate **89** to DNA does not lead to DNA compaction, as is observed with cationic lipids (Soto *et al* 2000). The fluorescent intensity of Hoechst 33258 increases upon binding to DNA, and therefore has found application as a fluorescent DNA probe (Gago *et al* 1988). Potentially, the intracellular study of complexes between conjugate **89** and plasmid DNA may be achieved using fluorescent microscopy, though currently its efficacy as a non-viral gene delivery tool has not been published.



Hoechst 33258



89

Figure 59. Structure of Hoechst 33258 conjugate **89** – a fluorescent DNA-binding lipid designed for gene delivery

Synthesis of fluorescent alkylspermine-based lipopolyamines incorporating lipophilic naphthalimide fluorophores

Lucifer yellow **90** (Fig. 60) is a fluorescent dye used in the study of neurones. Electrically coupled neurones allow exchange of Lucifer yellow where other cells do not, and are said to be "dye-coupled". Under intense illumination, Lucifer yellow dyes mediate cell death and therefore have the potential of forming the basis of selective tumouricidal agents (Chang *et al* 1999). These dyes are substituted 4-amino-1,8-naphthalimides that offer high photostability and fluorescence and, as well as fluorescent labels, have found application in liquid crystal displays and brightners in paints and plastics (Grabtchev *et al* 1996, Chang *et al* 1999). Professor David Lewis and co-workers (University of Wisconsin Eau-Claire) have prepared highly fluorescent lipophilic 1,8-naphthalimides (including **91** and **92**, Fig. 60, Chang *et al* 1999). Lipophilic fluorophores such as BODIPY (Fig. 53 and see also Chapter 1) have been incorporated into oligonucleotide delivery systems (Marcusson *et al* 1998). As part of our studies in fluorescent vectors for non-viral gene delivery, we were interested in incorporating a variety of fluorophores, including lipophilic moieties. To this end, Lewis generously supplied us with naphthalimides **91** and **92**.

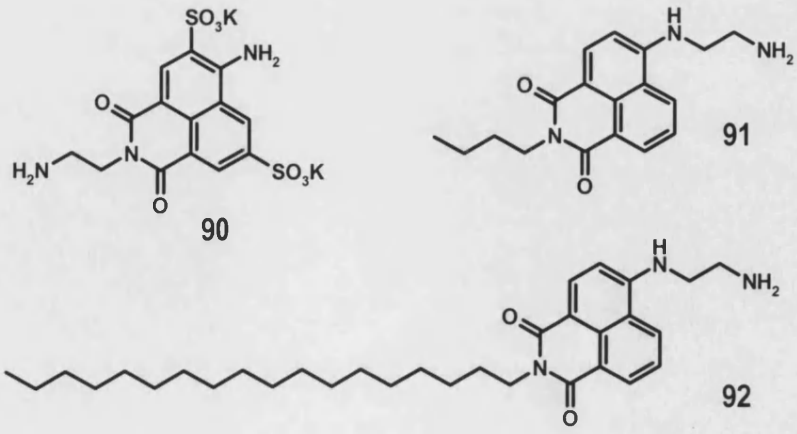


Figure 60. Naphthalimide-based fluorescent dyes: Lucifer Yellow ethylenediamine (potassium salt) **90** and Lewis naphthalimides **91** and **92**

In order to synthesise fluorescent naphthalimide lipopolyamine probes we proposed a short-chain linkage from the steroid to the fluorophore in analogy with our first series of fluorescent lipopolyamines, i.e. using the 3α-hydroxyl functionality of bile acid amide **50**. *N*-Alkyl-4-amino-1,8-naphthalimide dyes **91** and **92** contain a primary amine functional group. The design of fluorophores **93** and **94** allows us to tether these amines to the lipid scaffold via a succinate chain, giving a linker between lipid and fluorophore (Fig. 61) comparable with our designed 5-aminovalerate esters described earlier in this chapter.

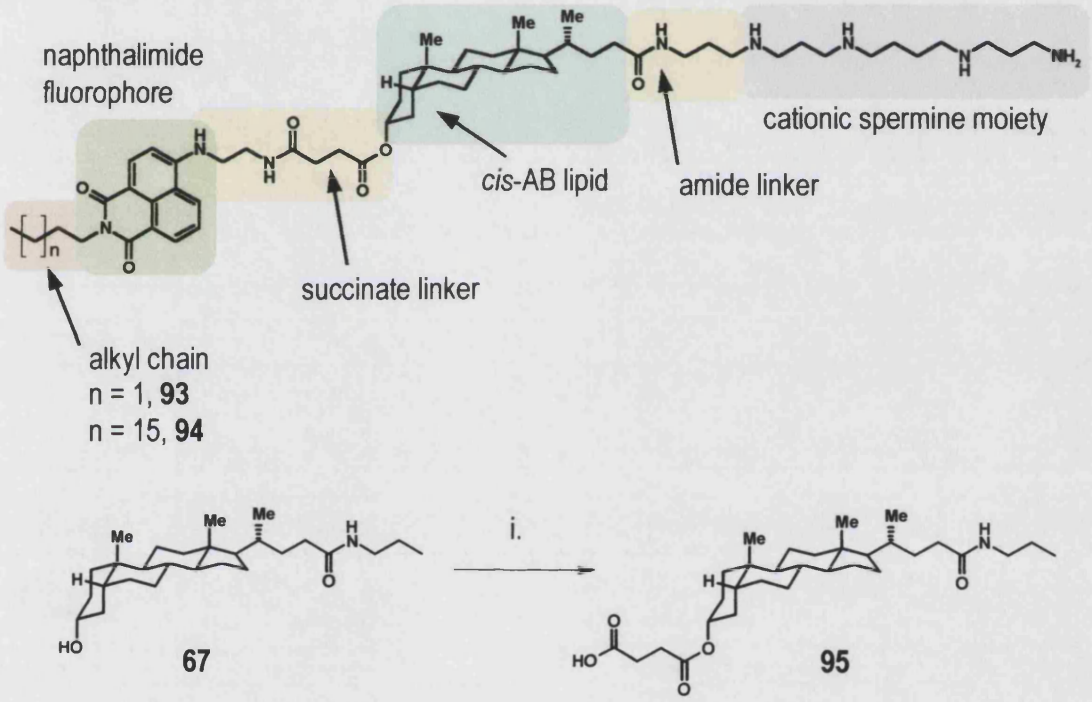


Figure 61. Structures of designed target naphthalimide-based lithocholic acid alkylspermine conjugates **93** and **94** and model studies of hemisuccinate ester **95** formation

i. Succinic anhydride, pyridine, 110 °C, 20 h

Preliminary model studies (Fig. 61) demonstrated that hemisuccinate esters such as **95** could be prepared in CH_2Cl_2 from the secondary alcohol, succinic anhydride and one equivalent of pyridine. However, heating (110 °C) in neat pyridine was established to be a superior method, affording homogeneous hemisuccinates in good yields (typically 80-90 %). We then employed this procedure for generating the hemisuccinate ester of polyamine-lithocholic conjugate **50** so that naphthalimides **91** and **92** could be coupled through an amide of succinic acid mono ester. As fluorescent naphthalimides **91** and **92** were precious materials, a linear synthesis also suited this small-scale work.

Alcohol **50** was heated under reflux in pyridine with an excess of succinic anhydride to afford the hemisuccinate ester **96** (Fig. 62). The steroidal 3β-proton in **96** was deshielded upon esterification from 3.57-3.66 ppm in **50** to 4.69-4.80 ppm, a typical observation in ^1H NMR spectra following the formation of esters. Carboxylic acid **96** was aminated with naphthalimide **91** (used without further purification), to afford the *N*-butyl naphthalimide derivative **97** which was purified over silica gel (R_f 0.62, CH_2Cl_2-MeOH 10:1 v/v). This naphthalimide **97** displayed the appropriate accurate mass data ($C_{79}H_{127}N_8O_{14}$ requires 1411.9472 (MH^+), found 1411.9495). In addition, up to four losses of 100 m/z were observed for conjugate **97**, indicating poly-Boc fragmentation.

Analogous treatment of carboxylic acid **96** with amine **92** afforded highly lipophilic *N*-octadecyl naphthalimide derivative **98** which was purified over silica gel (R_f 0.80, CH_2Cl_2-MeOH 10:1 v/v). Conjugate **98** displayed the appropriate accurate mass data ($C_{93}H_{155}N_8O_{14}$ requires 1608.1663 (MH^+), found 1608.1648). Again, up to four losses of 100 m/z were observed, inferring an intact poly-Boc protected polyamine moiety is present in conjugate **98**. Naphthalimide conjugate **98** is a novel fluorescent lipopolyamine bearing a C18 lipid chain and a steroid moiety.

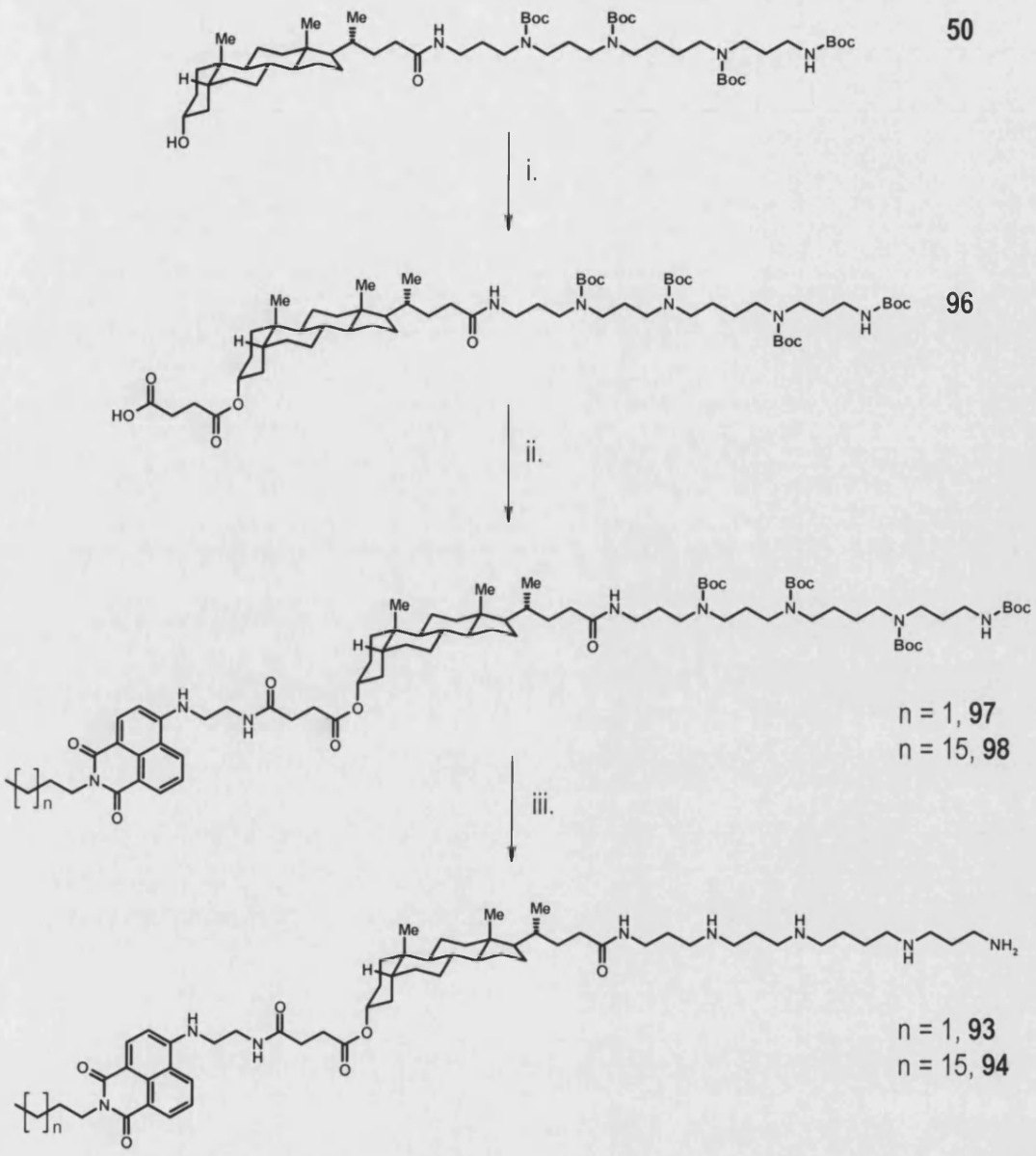


Figure 62. Synthesis of naphthalimide-labelled lipopolyamines **93** and **94**

i. Succinic anhydride, pyridine, 110 °C, 16 h; ii. **91** (n = 1) or **92** (n = 15), EDC, HOBt, CH_2Cl_2, 25 °C, 16 h;

iii. TFA-CH_2Cl_2 (1:9), 25 °C, 3 h

Compounds **97** and **98** were then treated with TFA-CH_2Cl_2 (1:9 v/v) in order to deprotect these polyamine conjugates. Upon addition of TFA to these solutions in anhydrous CH_2Cl_2, the colour turned from vibrant fluorescent yellow to orange, and we concluded that the 4-aminonaphthalimide moiety has a degree of pH-dependent fluorescence. These poly-Boc deprotections were monitored by TLC (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) until all

fluorescent (visualised at 366 nm excitation) and ninhydrin stained spots had moved to the baseline, typically after 3 h. Deprotected polyamine conjugates **93** and **94** were purified by semi-preparative RP-HPLC, detecting the naphthalimide chromophore at 430 nm (MeCN-0.01 % aq. TFA 9:1 v/v).

Fig. 63 shows a typical chromatogram following the TFA deprotection of poly-Boc spermine conjugate **97**. The retention time (t_R 19.5 min) of the poly-Boc protected starting material **97** was confirmed by co-injection. The first large peak (t_R 5.2 min) was collected and assumed to be the desired product **93** (the major product, a tetraammonium salt, the most polar component and therefore eluting first under reversed-phase chromatographic conditions). This product afforded green fluorescence when visualised at 366 nm, and strongly stained red with ninhydrin indicating that both fluorophore and polyamine were present. The FAB^+ mass spectrum supported the conclusion that these moieties were present in the same molecule (**93**, $C_{59}H_{94}N_8O_6$ requires 1010, found 1011 (MH^+)) and polyamine fragmentation was observed (954 $[MH - (CH_2)_3NH_2]^+$; 883 $[MH - (CH_2)_4NH(CH_2)_3NH_2]^+$).

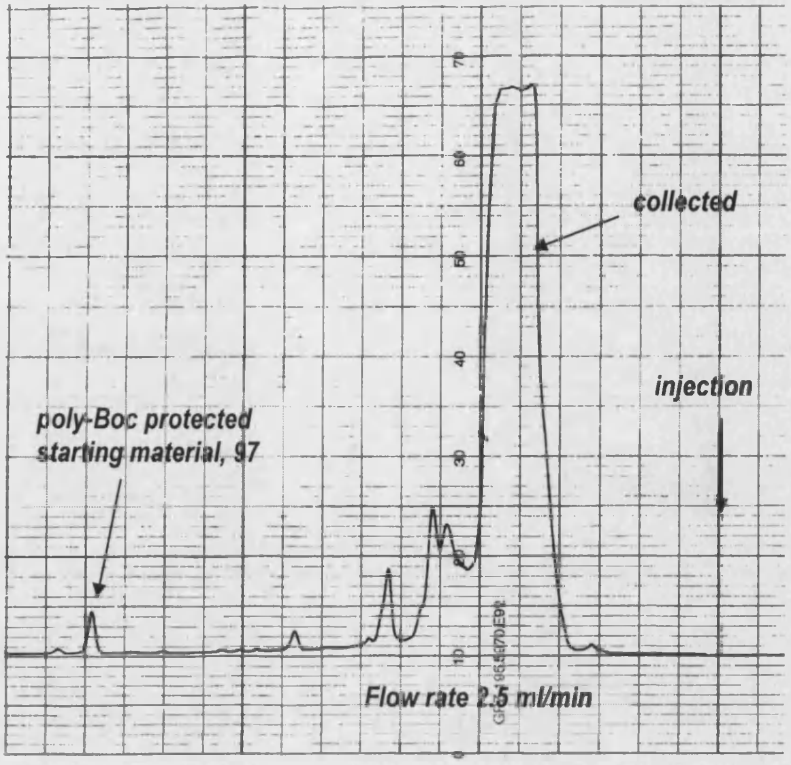


Figure 63. Semi-prep RP-HPLC chromatogram of poly-Boc deprotection of **97**

(Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v)

Fluorescent lipopolyamine **93** was freely soluble in water and spectroscopic analysis yielded similar fluorescence data (excitation λ_{max}: 460 nm, emission λ_{max}: 531 nm) to those published for naphthalimide **91** (Chang *et al* 1999, excitation λ_{max}: 450 nm, emission λ_{max}: 540 nm, measured in EtOH). The absorption spectrum (Fig. 64, λ_{max}: 453 nm, ε_{max} = 1.3 x 10^4) is similar to published values for 4-aminonaphthalimides in EtOH (Chang *et al* 1999, λ_{max}: 434 nm, ε_{max} = 1.2 x 10^4). These spectroscopic data confirm our lipopolyamine conjugate **93** as a fluorescent tool and evidence of its DNA binding is discussed below in Chapter 4.

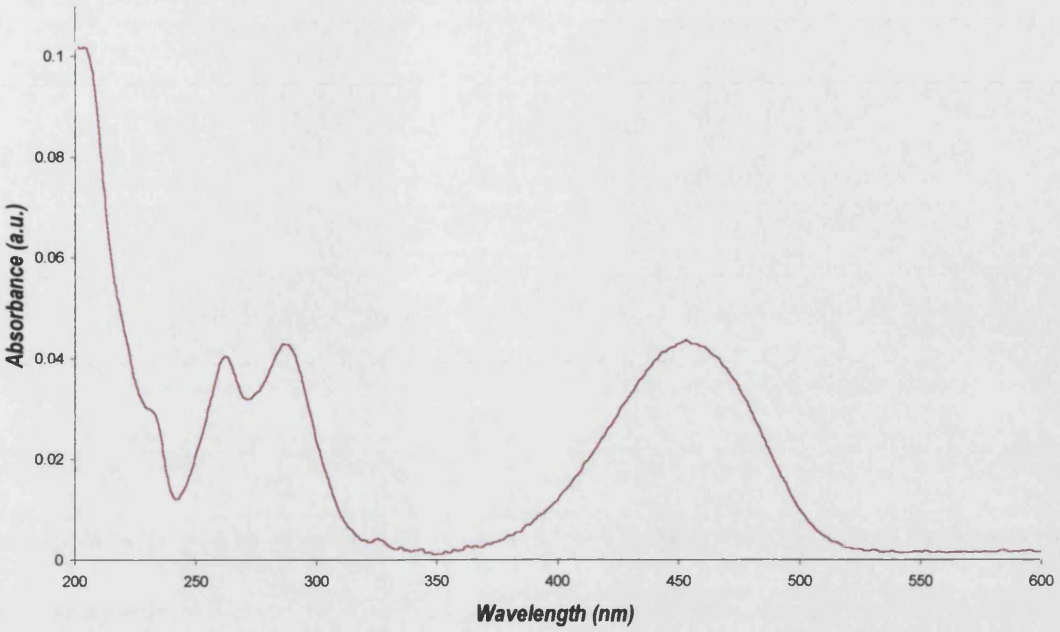


Figure 64. Normal absorption spectrum of **93** in H_2O

Fig. 65 shows sequential chromatograms for the collection of homogeneous product **94**. Mass spectral data confirm the collected component as the desired product **94** ($C_{73}H_{122}N_8O_6$ requires 1207, found 1208 (MH^+) 1230 ($M+Na^+$) and 1247 ($M+K^+$)) and polyamine fragmentation was again observed (1150 $[MH - (CH_2)_3NH_2]^+$; 1079 $[MH - (CH_2)_4NH(CH_2)_3NH_2]^+$). Conjugate **94**, bearing a single C18 lipid chain substituted fluorophore gave slightly shifted fluorescence (excitation λ_{max}: 450 nm, emission λ_{max}: 530 nm) and absorption (similar spectrum to that of **93**, λ_{max}: 439 nm) data, from which we concluded that these naphthalimides may be spectroscopically sensitive to hydrophobic environments. These properties may be useful for monitoring the intracellular status of DNA-lipopolyamine complexes, as many fluorophores change their fluorescence profile upon dissociation from DNA (Dougherty and Pigram 1982) e.g. ethidium bromide (Geall *et al* 1999).

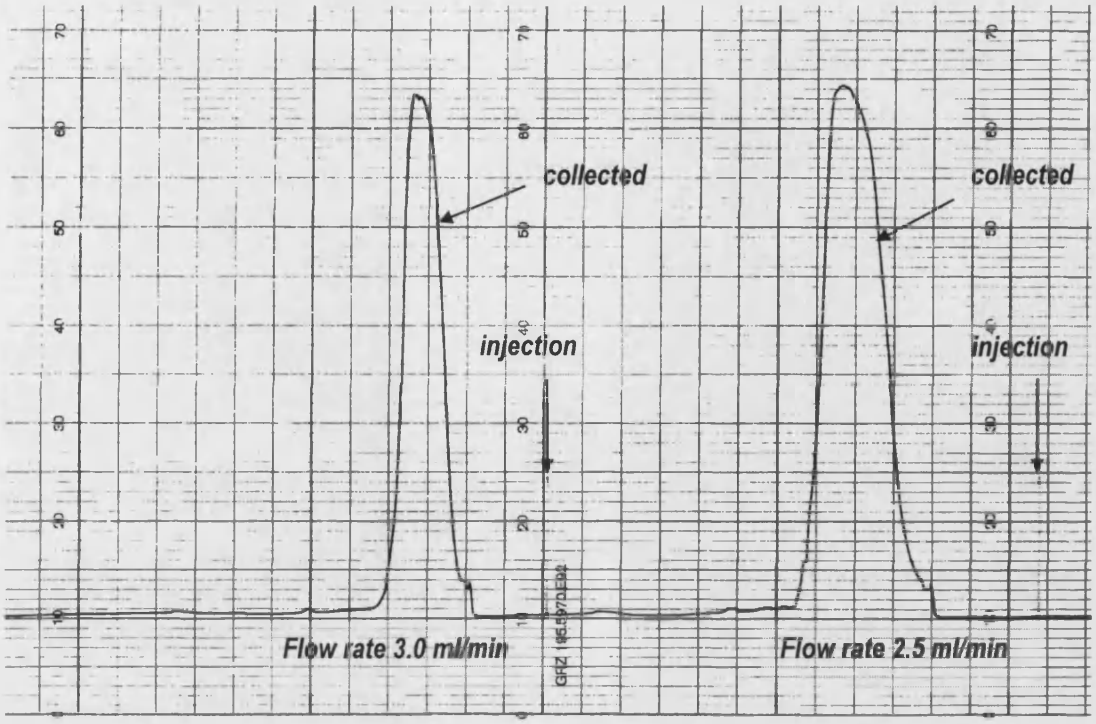
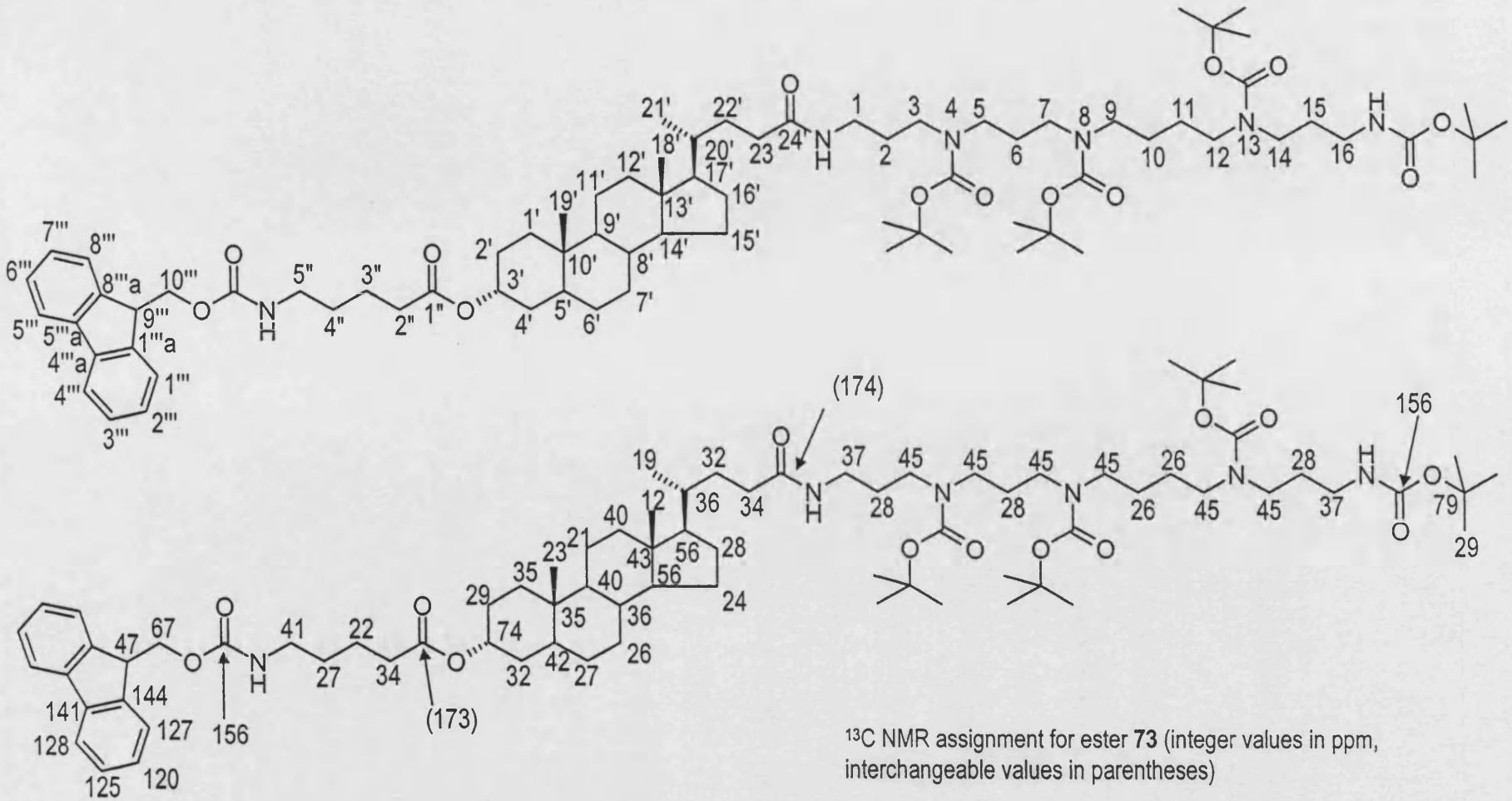


Figure 65. Semi-prep RP-HPLC sequential chromatograms of poly-Boc deprotection of **98** (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v)

Nomenclature and NMR assignment of key Fmoc protected intermediates

In this chapter, we report the design and synthesis of fluorescent lipopolyamines based on *cis*- and *trans*-AB steroids. The ^{13}C NMR spectra for the key spermine equivalents **73** and **77** have been rigorously assigned. By way of conclusion to this synthetic chapter, we present the nomenclature and total, unambigous ^{13}C NMR spectroscopic assignments of crucial precursors to our fluorescent lipopolyamines probes. The Boc-deprotected fluorescent lipopolyamines interact with DNA as discussed and characterised in Chapter 4.

Numbering system for N^4,N^8,N^{13},N^{16}-(tetra-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester **73**

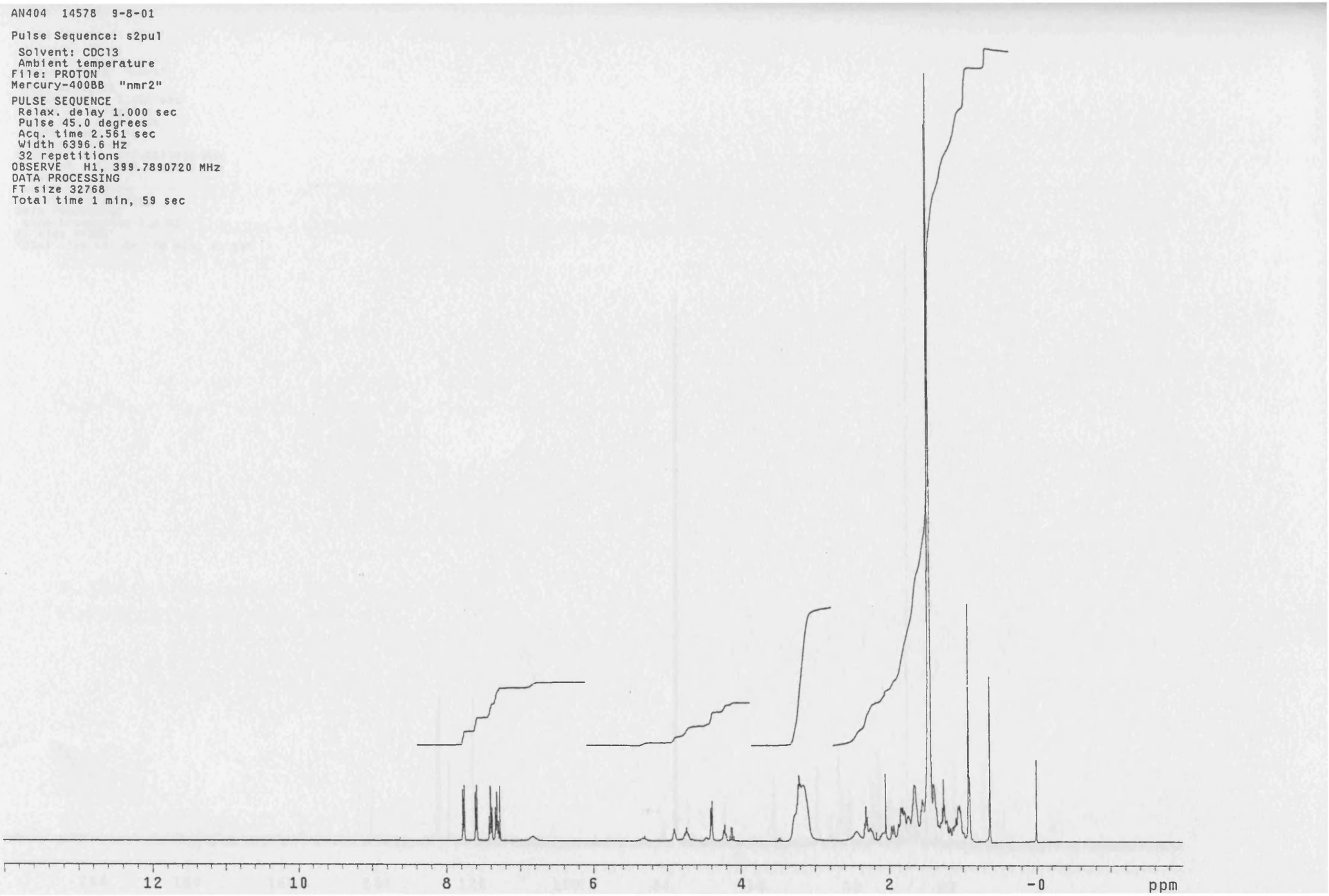


^{13}C NMR assignment for ester **73** (integer values in ppm, interchangeable values in parentheses)

AN404 14578 9-8-01

Pulse Sequence: s2pul
Solvent: CDCl3
Ambient temperature
File: PROTON
Mercury-400BB "nmr2"

PULSE SEQUENCE
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 2.561 sec
Width 6396.6 Hz
32 repetitions
OBSERVE H1, 399.7890720 MHz
DATA PROCESSING
FT size 32768
Total time 1 min, 59 sec



AN404 14579 9-8-01

Pulse Sequence: s2pul

Solvent: CDCl3
Ambient temperature
Mercury-400BB "nmr2"

PULSE SEQUENCE
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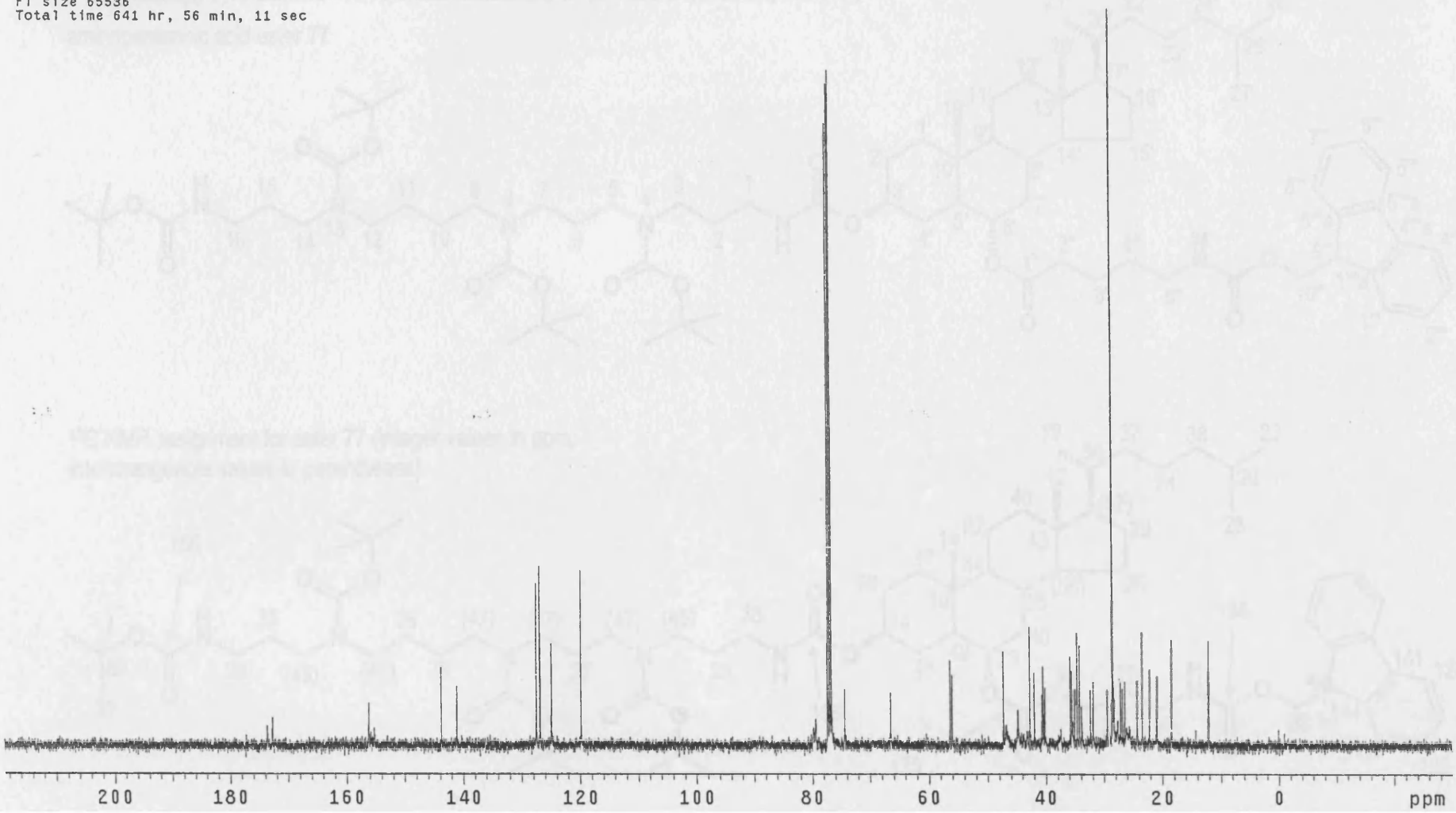
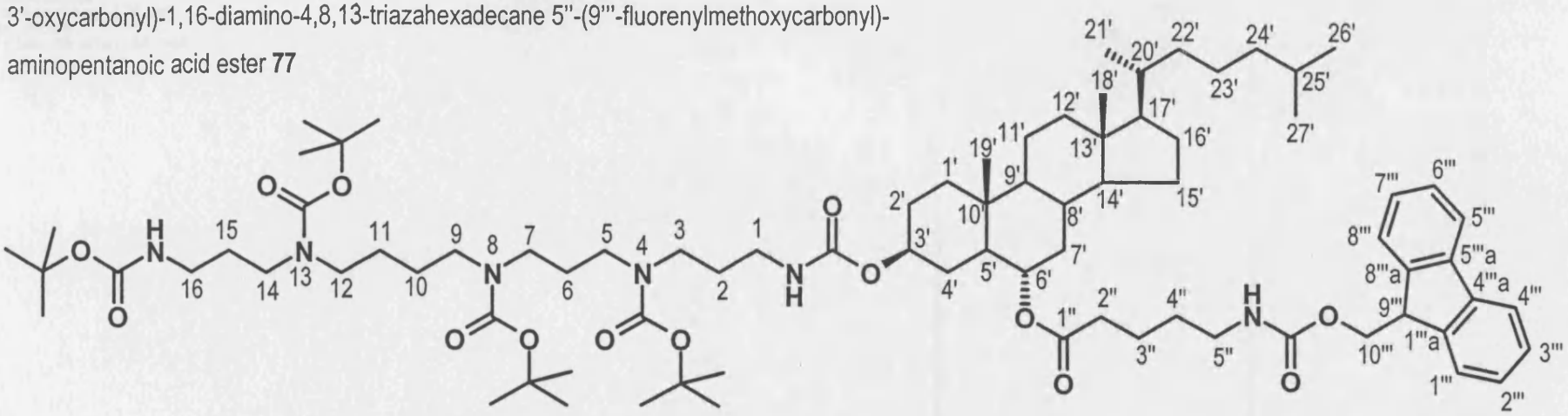
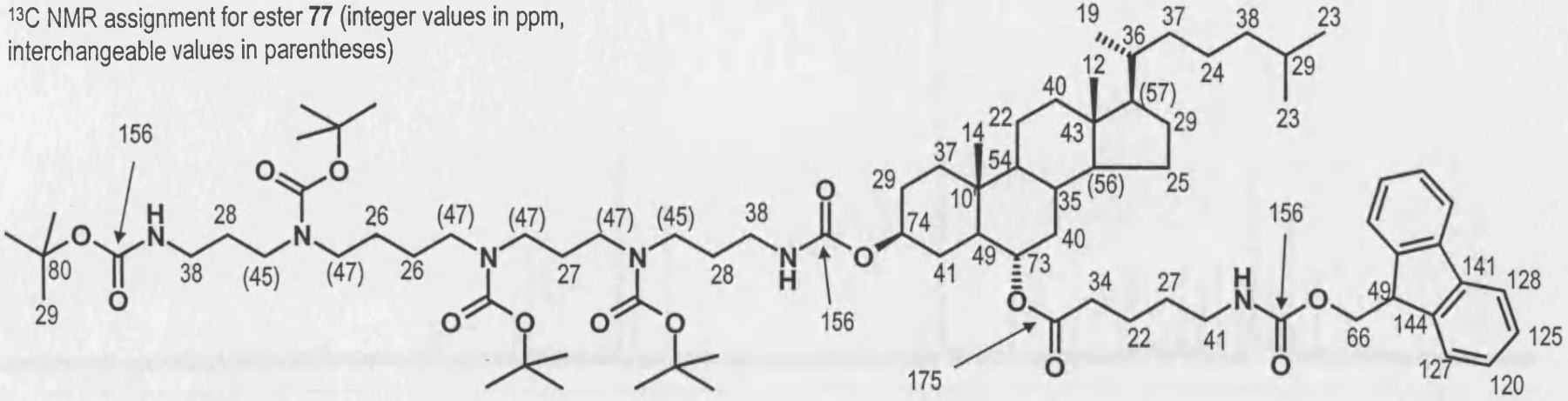
Pulse 54.2 degrees
Acq. time 1.199 sec
Width 25000.0 Hz
816 repetitions
OBSERVE C13, 100.5270516 MHz
DECOUPLE H1, 399.7911219 MHz
Power 43 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
Total time 641 hr, 56 min, 11 sec



Numbering system for N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester **77**



^{13}C NMR assignment for ester **77** (integer values in ppm, interchangeable values in parentheses)



AN420 15038 3-9-01

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Solvent: CDCl3
Ambient temperature
Mercury-400BB "nmr2"

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512 repetitions
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continuously on
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DATA PROCESSING
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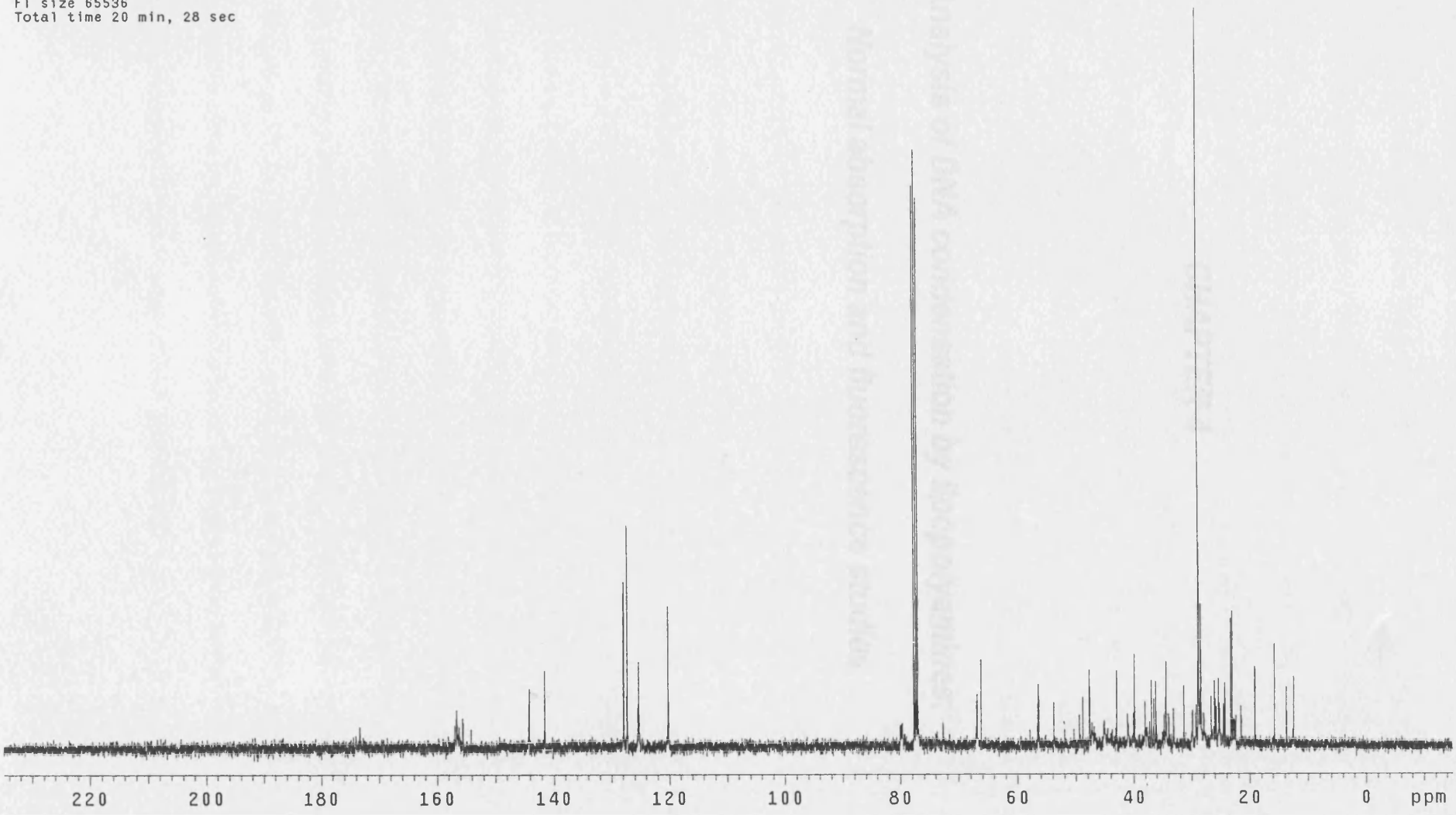


CHAPTER 4

Analysis of DNA condensation by lipopolyamines:

Normal absorption and fluorescence studies

Analysis of DNA condensation by lipopolyamines

Polyamine-DNA interactions have been extensively studied in the literature in order to attempt to understand the biological roles of polyamines. Natural and synthetic polyamines (such as spermine **1** 3.4.3, spermidine **2** 3.4 and thermine **99** 3.3.3 norspermine) are known to mediate a number of structural changes in DNA, such as the B→Z transition (Minyat *et al* 1978, Dickerson *et al* 1982, Thomas and Messner 1988) and may adopt a variety of binding orientations (Rodger *et al* 1995, Cohen 1998). Methods for the study of polyamine binding include NMR (Onash *et al* 1984) and molecular modelling (Liquori *et al* 1967, Adlam *et al* 1994, Rodger *et al* 1995). However, binding studies using these techniques are limited to relatively short sequences of duplex DNA (up to around 20 base pairs). Other techniques include X-ray crystallography (Drew and Dickerson 1981, Dickerson and Drew 1981), and recently, Raman spectroscopy (Deng *et al* 2000).

Although polyamines have weakly UV-visible absorbing chromophores, absorption spectroscopy may be used to study changes of "signature" DNA profiles upon polyamine binding. Variations of absorption spectroscopy include normal absorption, circular dichroism (CD, Parkinson *et al* 2000) and linear dichroism (LD, Rodger *et al* 1994). Although the aromatic hetereocyclic bases of DNA do not fluoresce, fluorescence spectroscopy is an available technique, provided a suitable fluorescent molecular probe is used. Examples of such probes are ethidium bromide (EtBr) **100** (a DNA intercalating agent, Dougherty and Pigram 1982) and Hoechst 33258 (a groove binder, see Chapter 3 Fig. 59, Moon *et al* 1995) and these may be displaced by polyamines, causing a change in the fluorescence (Patterkine and Ganesh 1999, Geall and Blagbrough 2000b). Normal absorption and fluorescence, using EtBr **100** as a DNA probe, are the basis of the spectroscopic studies reported here for the assessment of polyamine-DNA interactions in the context of non-viral gene delivery.

Polyamine-DNA binding studies

We have studied the DNA-binding of three simple polyamines, spermine **1**, spermidine **2** and thermine **99** (Fig. 70) using an EtBr **100** displacement assay (Geall and Blagbrough 2000b). This fluorescence technique, relies on polyamines displacing EtBr (Fig. 70), a cationic tricyclic intercalating agent. EtBr-DNA complexes are highly fluorescent (Geall and Blagbrough 2000b), but the fluorescence of unbound EtBr is quenched in aqueous media. By measuring the depreciation of fluorescence intensity as a function of increasing polyamine concentration, it is possible to assess the efficiency of polyamines in binding to DNA (Geall *et al* 1998b and 2000).

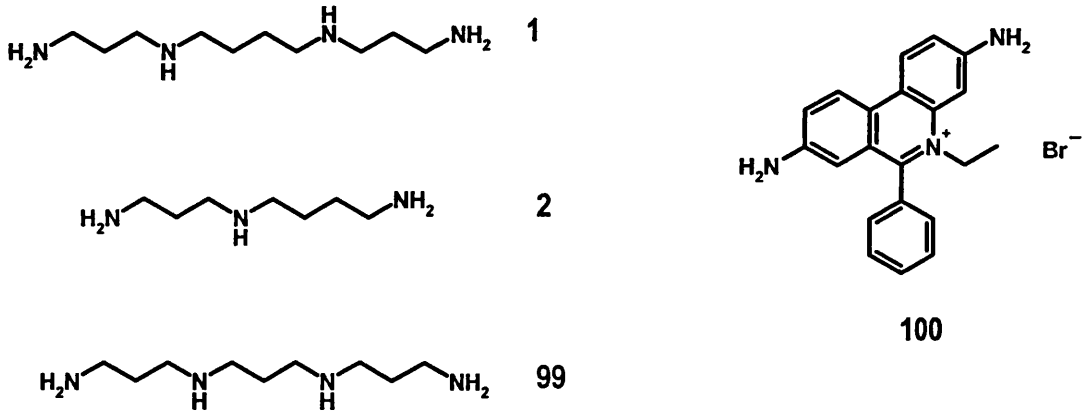


Figure 70. Structures of polyamines spermine **1**, spermidine **2**, thermine **99** and ethidium bromide **100**

EtBr emits at 600 nm, upon excitation at 546 nm, with a higher quantum yield when bound to DNA (Geall and Blagbrough 2000b). However, when the bases of EtBr bound-DNA are excited at 260 nm, fluorescence emission is observed at 600 nm. This energy transfer from the DNA bases to the intercalated EtBr allows us to measure only the intercalated EtBr with an approximately 10-fold increase in sensitivity over direct EtBr excitation at 546 nm and is therefore the method of choice (Gershon *et al* 1993).

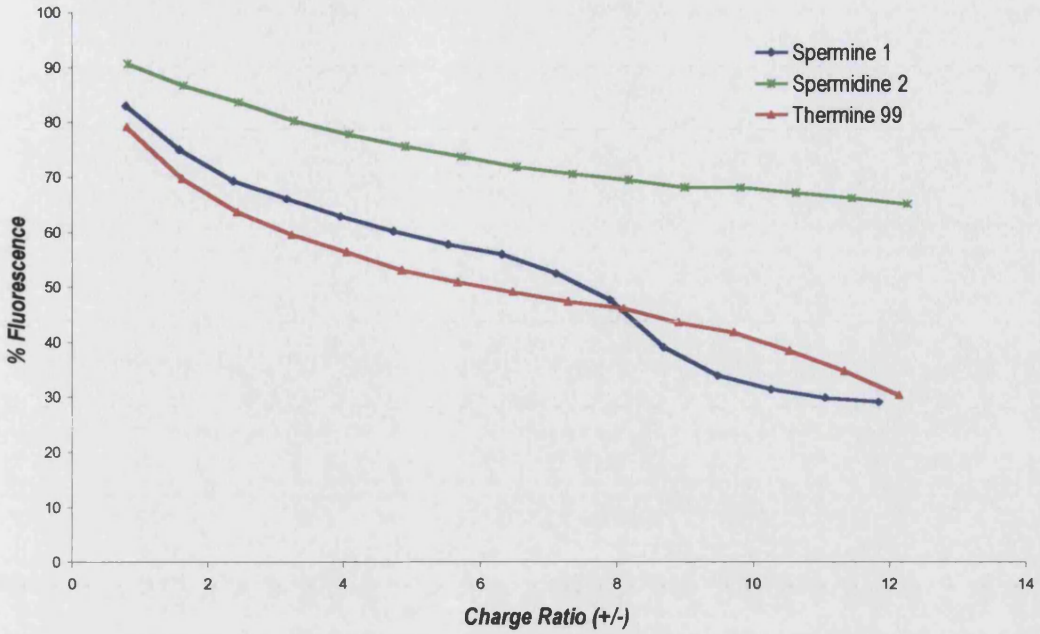


Figure 71. EtBr displacement profiles of spermine **1**, spermidine **2** and thermine **99**: calf thymus DNA (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

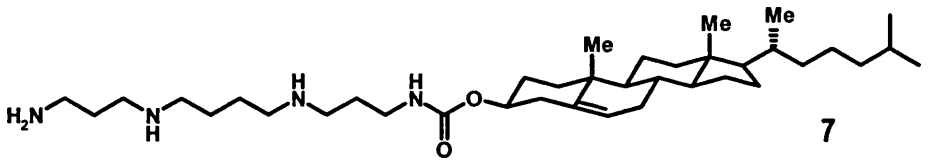
The displacement of EtBr was measured and plotted as a function of ligand ammonium:DNA phosphate ratio (charge ratio, Felgner *et al* 1997, Fig. 71). Spermine **1** with a net positive charge of 3.8 at pH 7.4 displaces EtBr with greater efficiency than spermidine **2** with 2.5 positive charges (Geall *et al* 1999). Thermine **99** binds with a similar affinity to spermine **1**, though the data are not identical, a reflection of the difference in the central methylene spacing reducing from four in spermine to three in thermine (sometimes called norspermine, see Fig. 70). Polyamine-DNA binding has been shown to be a function of charge and the regiochemical distribution of polyammonium ions (Geall *et al* 1998b and 1999) and these results are in agreement with this.

Polyamines are known to condense DNA into toroidal shaped particles (Hud 1995, Hud *et al* 1995) and these particles have recently been imaged using AFM and electron microscopy (Lin *et al* 1998). EtBr displacement is also a measure of DNA condensation by polyamines, as charge neutralisation effects changes in the intercalation binding-sites making them

unfavourable for EtBr intercalation. DNA bending has recently been observed by LD, during the condensation process (Rodger *et al* 2000). DNA condensation is predicted to occur when around 90 % of the DNA polyanionic charge has been neutralised (Manning 1978, Wilson and Bloomfield 1979). In the assays reported here, DNA condensation is not complete around a 12-fold cationic charge excess of polyamine. We conclude that DNA condensation by simple polyamines is an inefficient process. These results are in agreement with literature findings (Geall *et al* 1998b).

DNA condensation for gene delivery: cationic polymers and cationic lipids

DNA condensation is a key first step in the transfection process, during which cells internalise DNA particles. Therefore, we have used the EtBr displacement to assess poly-L-lysine (PLL, Fig. 72) and polyamine cholesteryl carbamate-mediated DNA condensation and particle formation.



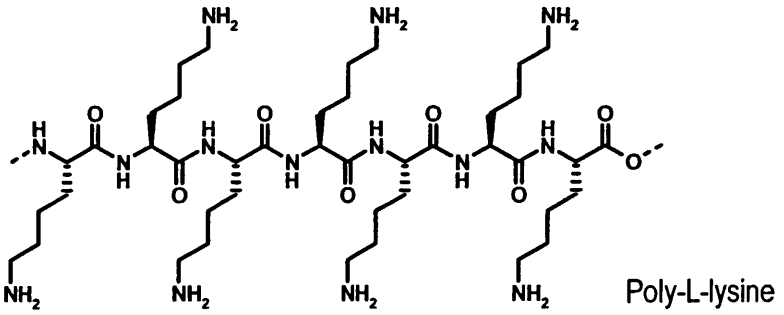


Figure 72. Structures of cholesteryl carbamate **7** and poly-L-lysine

In Fig. 73, we show the EtBr displacement assay of spermidine mimic cholesteryl carbamate **7** (Fig. 72) measured at 20 mM NaCl ("low salt") and at 150 mM NaCl ("high salt", the physiological salt concentration). DNA-binding experiments are frequently reported at low salt concentrations in the literature (commonly 20 mM NaCl) and are therefore important for the purpose of comparison. Polyamines and their conjugates afford salt dependent DNA-binding profiles, a reflection of the electrostatic nature of the interactions involved. At 20mM NaCl, DNA is condensed efficiently by carbamate **7**, with efficient displacement of EtBr at a charge ratio of around 1. That the EtBr fluorescence is not fully quenched indicates some EtBr is retained in the condensed particle, possibly due to aggregation of DNA condensates. The condensation process is less efficient at 150 mM NaCl possibly due to the significantly increased electrostatic competition.

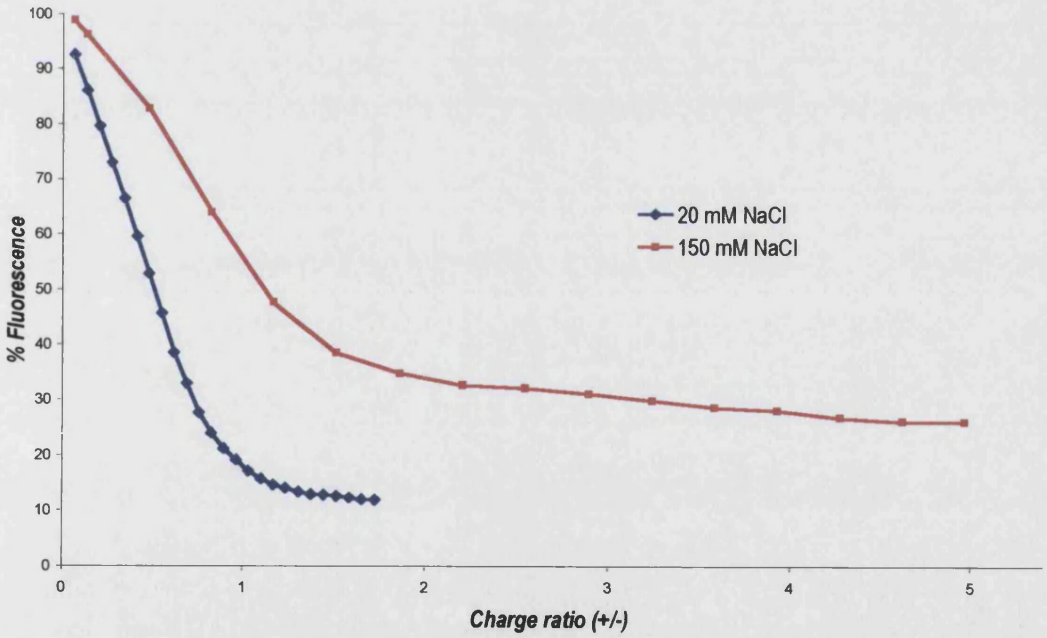


Figure 73. EtBr displacement profiles of cholesteryl carbamate **7**:
calf thymus DNA at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA) (blue)
and high salt (150 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA) (red)

In order to correlate EtBr displacement with particle formation (in order to show that EtBr displacement is related to a condensation mechanism and change in DNA structure), we have also conducted light-scattering studies. The normal absorption spectra of polyamine-DNA increases above 300 nm as the concentration of polycation is increased (Wilson and Bloomfield 1979, Patterkine and Ganesh 1999), a background light-scattering effect caused by the formation of particles of DNA.

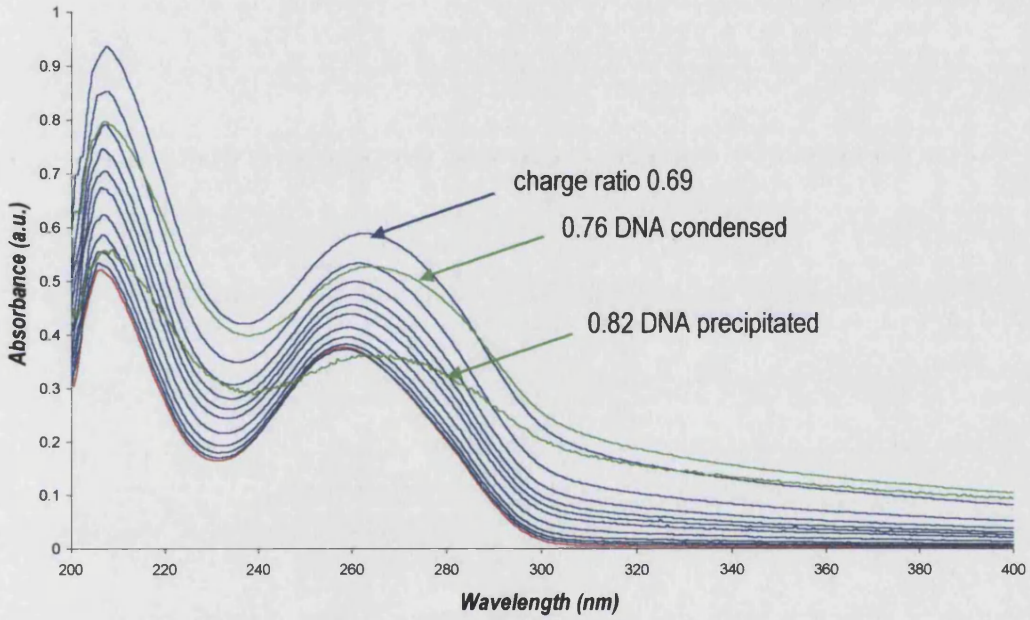


Figure 74. Normal absorption spectra of 60 μg calf thymus DNA (red) and 60 μg calf thymus DNA + increasing concentrations of cholesteryl carbamate **7** (blue) until DNA condensation and precipitation (green): low salt 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA

In Fig. 74, we show a changing normal absorption profile for calf thymus DNA upon increasing concentrations of cholesteryl carbamate **7**, measured at low salt. As drug load on the DNA increases, the profile intensifies and broadens across the spectrum. The band centred around 260 nm is composed of a complex number of transitions associated with the DNA bases. In B-form double stranded DNA the absorption is significantly less than the sum of the bases. This hypochromic effect (an effect leading to decreased absorption intensity) is

caused by π-π interactions between stacked base pairs. In Fig. 74, hyperchromicity (increase in absorbance) is observed for the band at 260 nm and is accompanied by a red shift, indications that polyamine conjugate **7** is binding to DNA. These observations are consistent with stages in a condensation mechanism. The extent of the DNA-base stacking is diminished by polyamine-induced DNA bending. The π-π interactions that are effective in lowering the absorption of DNA in the absence of polyamines are disrupted by conformational changes of the DNA upon polyamine binding. Therefore, a hyperchromic effect is observed in the normal absorption spectroscopy of DNA-polyamine complexes (Rodger *et al* 2000). The conformational changes and ultimately, DNA condensation upon polyamine binding, also account for the displacement of ethidium cations from intercalation sites in the fluorescence assay. Increasing the drug load eventually leads to DNA condensation (charge ratio 0.76) and further titration with cholesteryl carbamate **7** causes a decrease in absorption as condensed DNA aggregates precipitate, which is visible to the naked eye (charge ratio 0.82). Although these values for DNA condensation do not correlate well with the fluorescence assay values (at charge ratio 0.82, around 25 % EtBr is complexed to DNA), time between addition of aliquots of cholesteryl carbamate **7** was not rigorously controlled during the collection of absorption data. The size of condensed DNA particles is known to vary with time (Lee and Huang 1996). In the fluorescence assay, polyamine addition is separated by one minute intervals, a consistent equilibration time. As absorption studies are not as sensitive as fluorescence measurements, solutions are 10-fold more concentrated than for the fluorescence experiments and do not include EtBr. Having established the spectroscopy, we repeated this experiment, measuring absorbance at a fixed wavelength (320 nm), with the addition of cholesteryl carbamate **7** at timed intervals (Geall and Blagbrough 2000a, Geall *et al* 2000).

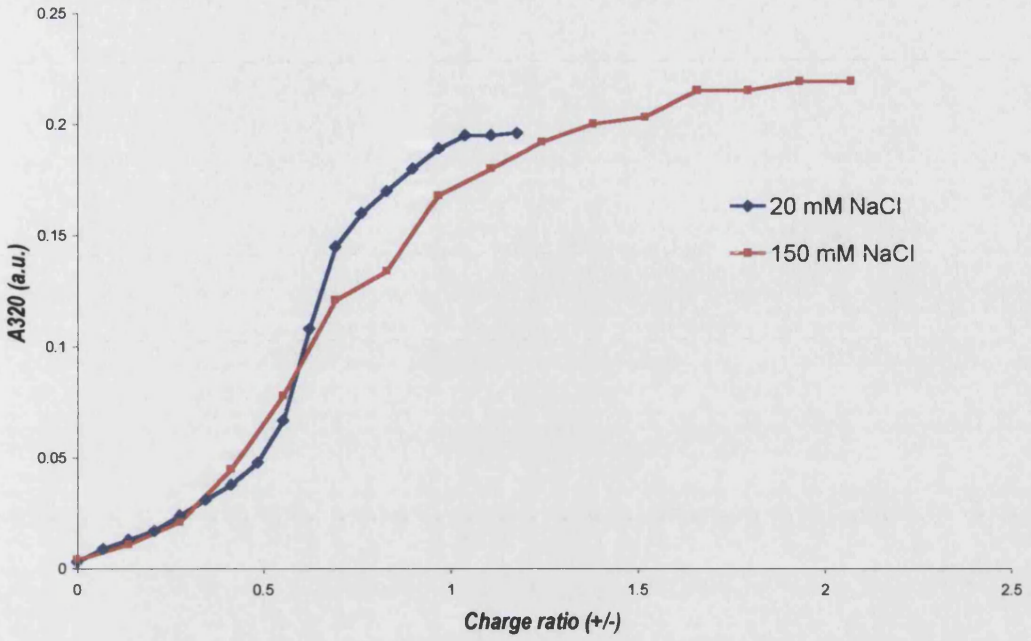


Figure 75. Light scattering profiles of cholesteryl carbamate **7**: calf thymus DNA at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA) (blue) and high salt (150 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA) (red)

Fig. 75 is the fixed wavelength (320 nm) light scattering assay for cholesteryl carbamate **7** binding to calf thymus DNA showing an increase in light scattered as a function of charge ratio. Particle formation occurs at both low and high salt concentrations, as predicted from the EtBr displacement assay. By superimposing the light scattering and fluorescence curves for cholesteryl carbamate **7** at low salt concentration (Fig. 76), it is possible to compare the binding of polyammonium ligand (displacement of EtBr) and DNA particle formation (light scattering). At charge ratios of around 1.1, the absorbance and EtBr displacement approach maxima indicating particle formation by DNA condensation is complete (though only within the time parameters of these experiments). However, the two systems described here are at different concentrations, and in the light scattering experiments, EtBr is absent. EtBr may impart different polyamine binding geometries, through control of the DNA solution structure.

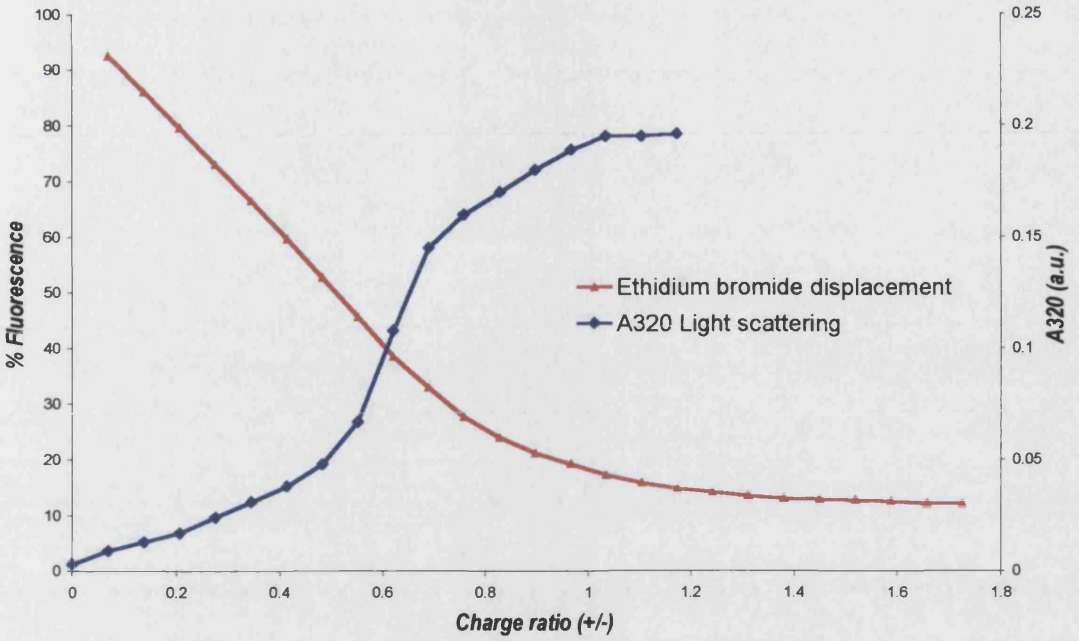


Figure 76. DNA Condensation and particle formation: superimposed EtBr displacement (red) and light scattering (blue) profiles of cholesteryl carbamate **7** with calf thymus DNA at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

Base-sequence preference of lipopolyamines and cationic polymers

We have investigated the binding of cholesteryl carbamate **7** and poly-L-lysine (PLL) to natural and synthetic sequences of DNA. Binding of these gene delivery agents to duplex alternating copolymers of complementary DNA bases is a measure of sequence preference. The EtBr displacement profiles for cholesteryl carbamate **7** binding to calf thymus DNA, poly[d(A-T)]$_2$ and poly[d(G-C)]$_2$ at low salt concentration are shown in Fig. 77. Calf thymus DNA (a mixed polymer of A-T (58 %) and G-C (42 %), Voet and Voet 1995) and poly [d(G-C)]$_2$ are condensed with similar efficiency. Condensation of poly[d(A-T)]$_2$ is a more efficient process than the other sequences studied. This is in agreement with other published studies (Hsieh et al 1995) where a preference of steroidal polyamines for A-T rich sequences was reported.

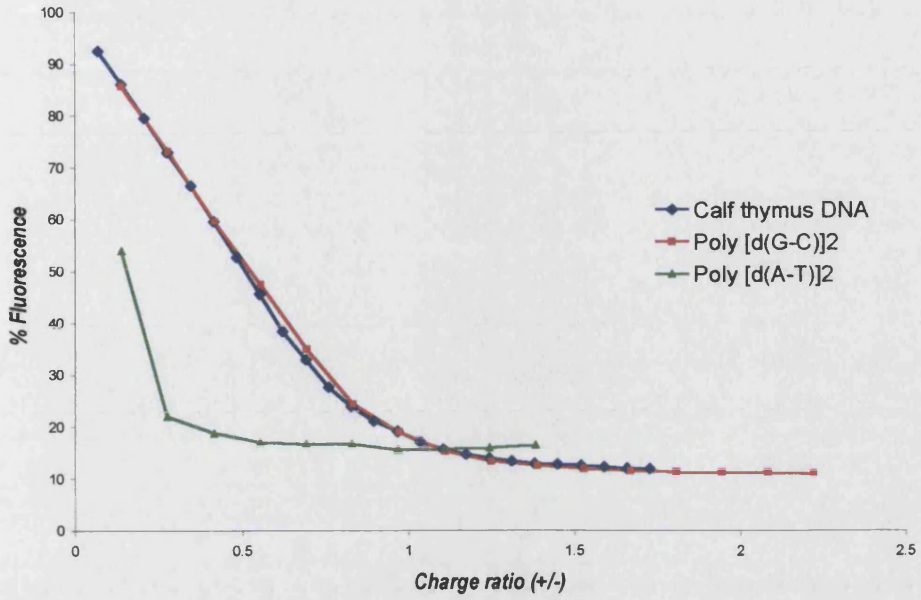


Figure 77. EtBr displacement profiles of cholesteryl carbamate **7** with natural and synthetic DNA: Calf thymus DNA (blue), poly $[d(G\text{-}C)]_2$ (red) and poly $[d(A\text{-}T)]_2$ (green) at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

Similar conclusions may be drawn from data acquired for the binding of a 255-mer PLL (Fig. 78) and are in agreement with the findings of Leng and Felsenfeld (1966). Salt concentration has less effect on efficient DNA condensation (cf. data for cholesteryl carbamate **7**, Fig. 73).

PLL is an efficient transfection vector and a mimic of histones conserving lysine-rich sequences in the condensation of DNA. PLL is commercially available in various average molecular weights and polydispersities. The influence of molecular weight on transfection activity of another cationic polymer polyethylenimine (PEI) has recently been reported (Godbey *et al* 1999a) indicating that higher average molecular weight PEI affords the highest transfection efficiency. Using the EtBr and light scattering assays, we investigated the condensation of calf thymus DNA for three lengths of PLL.

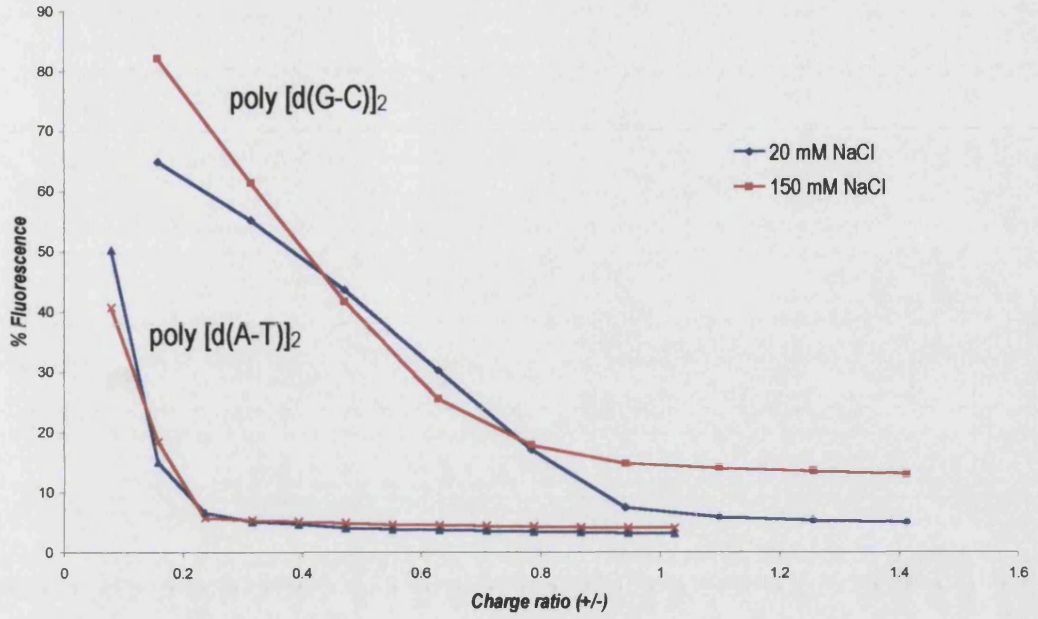


Figure 78. EtBr displacement profiles of 255-mer poly-L-lysine with natural and synthetic DNA: Calf thymus DNA, poly $[d(G-C)]_2$ and poly $[d(A-T)]_2$ at low salt (blue) (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA) and high salt (red) (150 mM NaCl buffer, 2mM HEPES, 0.05 mM

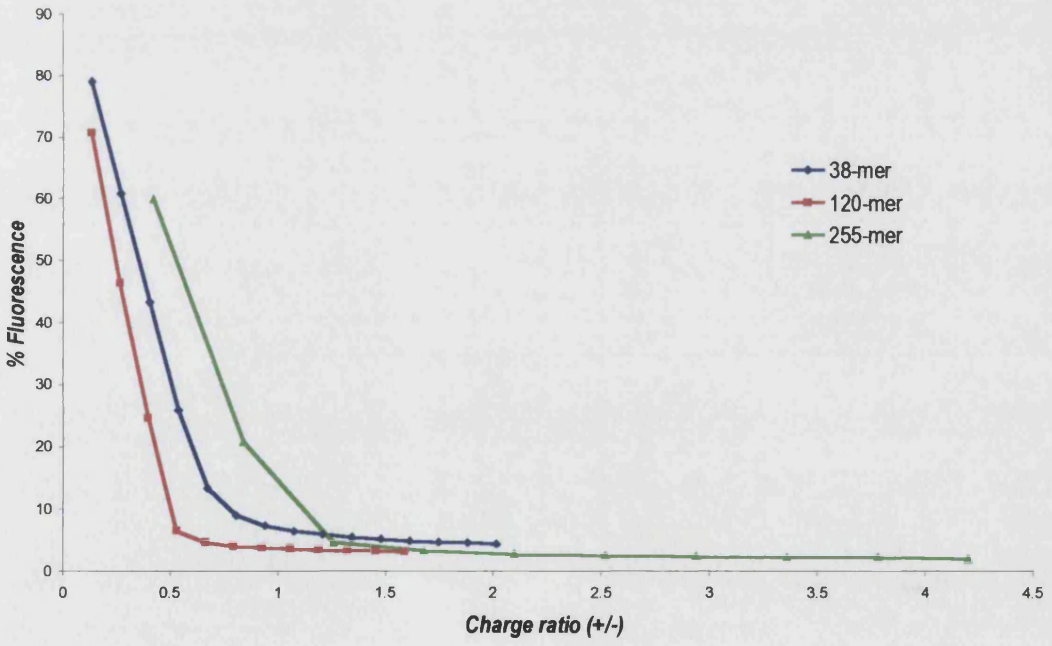


Figure 79. EtBr displacement profiles of 38-, 120- and 255-mer poly-L-lysine: calf thymus DNA at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

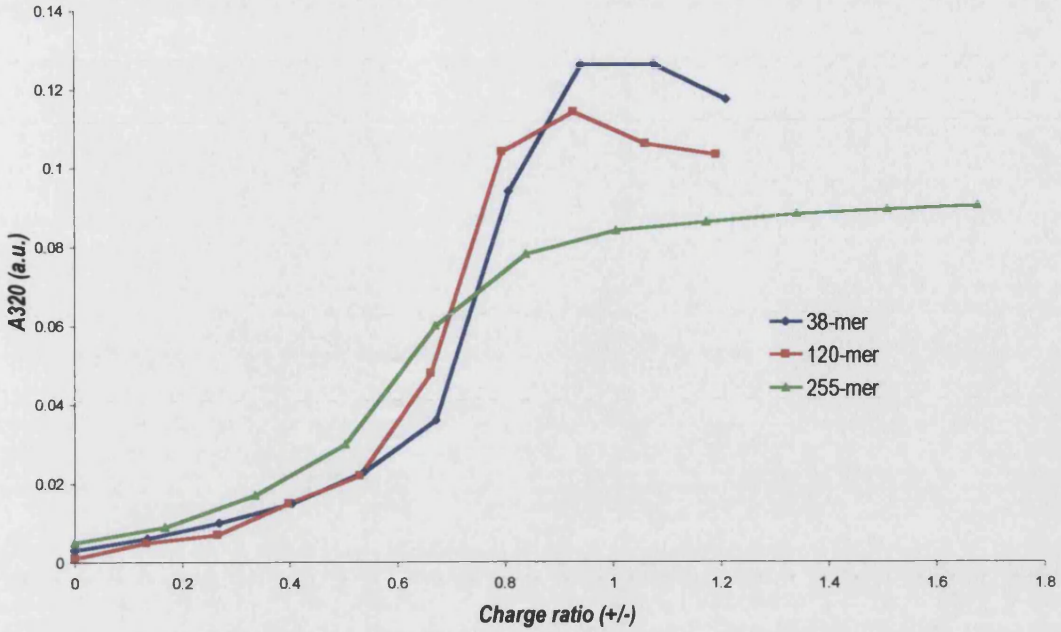


Figure 80. Light scattering profiles of 38-, 120- and 255-mer poly-L-lysine: calf thymus DNA at low salt (20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

Under low salt conditions, PLL 120 is the most efficient average length polymer for DNA condensation, as followed by EtBr displacement (Fig. 79) and light scattering (Fig. 80). Particle formation followed by precipitation was observed for PLL 120 at a charge ratio less than 0.9. PLL of all degrees of polymerisation tested are more efficient than cholesteryl spermidine mimic **7**. Similar results were recorded at the high salt concentration (Fig. 81 and 82).

In the context of gene delivery, cationic vectors have been shown to transfect cells at charge ratios above 1.0 (Miller 1998, Schwartz *et al* 1999). At these charge ratios, all lengths of PLL mediate DNA particle formation at physiological pH (7.4) and salt concentration.

It is reasonable to propose that larger particles will increase the angle of light scattering thereby elevating the apparent absorbance as less light is collected by the instrument. Under this assumption, particles formed at 150 mM NaCl are larger than those formed at only 20 mM NaCl. Aggregation of DNA condensates may account for this and the observed precipitation at

higher charge ratios. Aggregation of PLL-DNA and polyamine-DNA complexes is consistent with electron microscopy studies revealing large (2000 nm diameter) PLL-DNA structures (Tang and Szoka 1997). Furthermore, the amount of light scattered by lipopolyamine carbamate **7**-DNA complexes is greater than cationic polymer PLL-DNA complexes at both salt concentrations used here (see Fig. 75), however, a higher charge ratio is required for cholesteryl carbamate **7**. In scanning force microscopy studies, Transfectam (DOGS, a di-C18 chain lipopolyamine spermine mimic, Fig. 83) was shown to condense DNA into larger particles than polyethylenimine (PEI, a cationic polymer, Fig. 83) at the same charge ratio (Dunlap *et al* 1997).

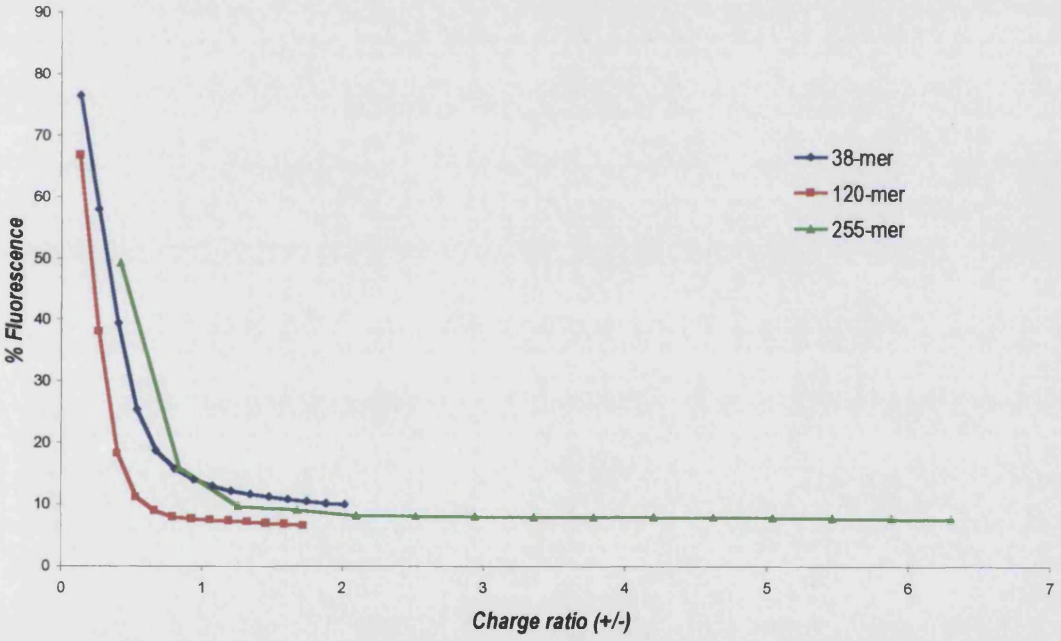


Figure 81. EtBr displacement profiles of 38-, 120- and 255-mer poly-L-lysine: calf thymus DNA at high salt (150 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

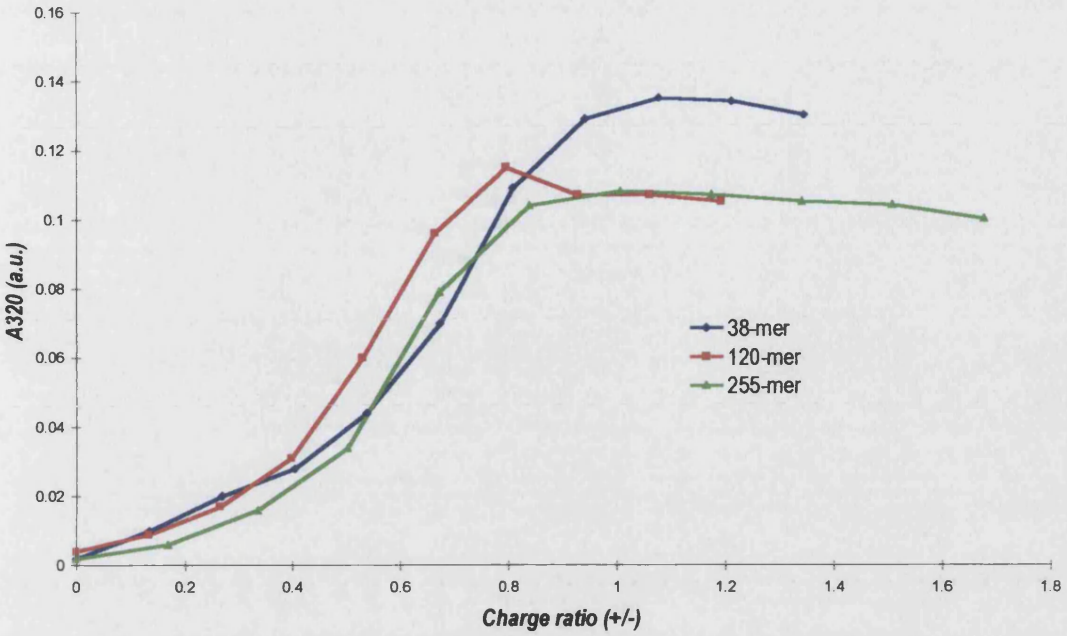
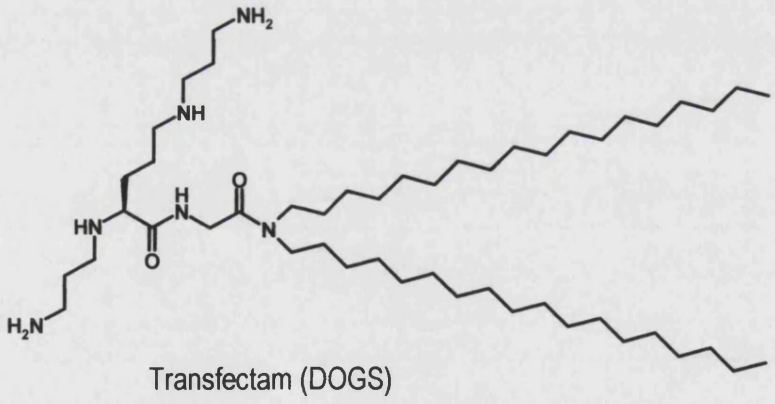
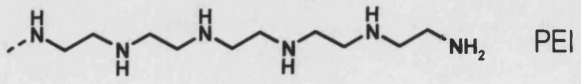


Figure 82. Light scattering profiles of 38-, 120- and 255-mer poly-L-lysine:

calf thymus DNA at high salt (150 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)



Transfectam (DOGS)



PEI

Figure 83. Structures of the lipopolyamine Transfectam and the linear cationic polymer PEI

Monocationic steroidal carbamates such as DC-Chol **26** (Chapter 2) are known to be useful tools for the transfection of cells *in vitro* (Gao and Huang 1991). This biological activity in some part relies on interactions between the cationic lipid and DNA. Using fluorescence and absorption spectroscopic techniques, we have made an initial assessment of these interactions between monocationic ergosteryl carbamate **33** (Fig. 84) and a natural sequence - calf thymus DNA. Fig. 84 shows a normal absorption curve of calf thymus DNA that changes upon the addition of ergosteryl carbamate **33**. The spectrum is observed to broaden and intensify especially at the 265 nm band and background above 300 nm. This is the same effect observed for the polyamine cholesteryl carbamate **7** described earlier and is also consistent with DNA condensation and light scattering through particle formation. Although ergosteryl carbamate **33** is a conjugated diene, it has a weakly absorbing chromophore which at the dilute concentrations reported here, does not interfere significantly with the normal absorption spectrum observed. We concluded that carbamate **33** is effecting a structural change on the DNA.

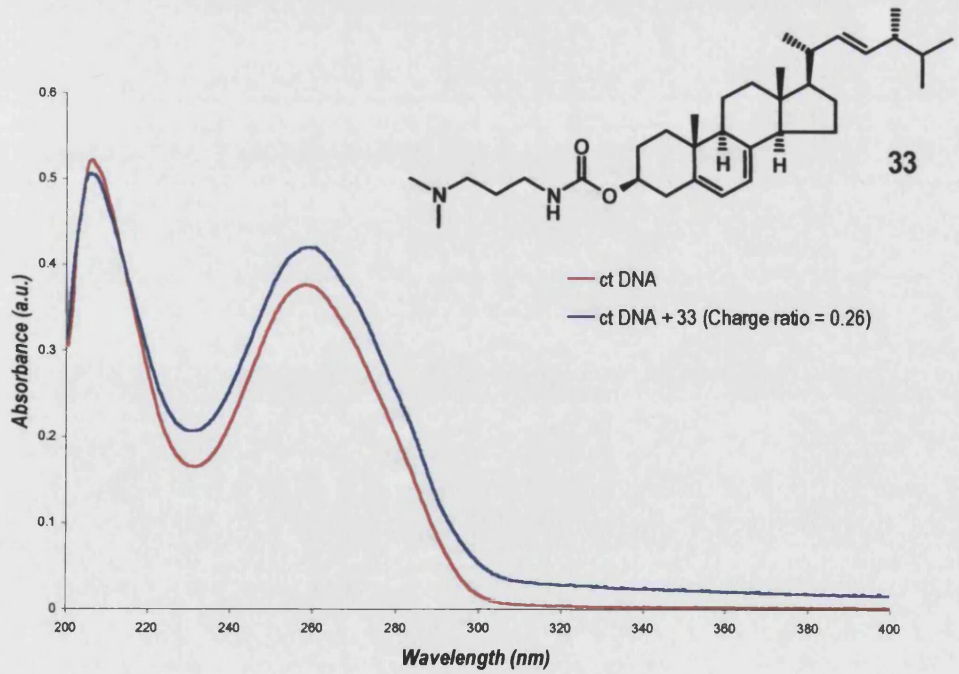


Figure 84. Structure of ergosteryl carbamate **33** and normal absorption spectra of 60 μg calf thymus DNA (red) and 60 μg calf thymus DNA + 3 μg carbamate **33** (blue) at 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA

Armed with these data, ergosteryl carbamate **33** was assayed for EtBr displacement which showed that carbamate **33** does mediate DNA condensation at low salt (Fig. 85) with more efficiency than the natural polyamine spermine **1**. DNA condensation is complete at a charge ratio of around 6 (90 % of EtBr displaced). This is less efficient than the lipopolyamines or cationic polyamines assayed in this work - this is in agreement with the literature that describes DNA condensation as a function of, in one measure, cationic charge at physiological pH, although lipophilicity and lipid shape are also parameters (Geall *et al* 1998b, 1999, 2000).

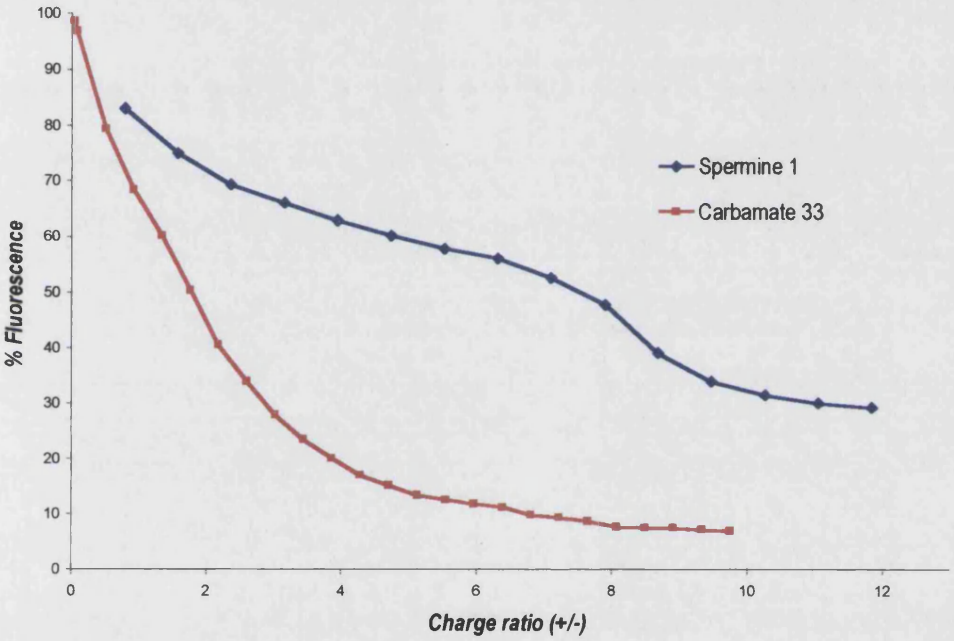


Figure 85. EtBr displacement profiles of ergosteryl carbamate **33** compared to spermine **1**.

(Calf thymus DNA, 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

DNA binding studies of cis-*AB and* trans-*AB steroidal lipopolyamines*

In addition to DNA binding affinity, polyamine-steroid conjugates have recently been shown to exhibit antimicrobial activity (Kikuchi *et al* 1997, Kim *et al* 2001). These steroidal lipopolyamines have been designed as mimics of squalamine **101** (Fig. 86), a powerful antimicrobial agent obtained naturally from the dogfish shark (*Squalus acanthias*, Kim *et al* 2001). Squalamine **101** is a steroidal spermidine derivative, hydroxylated at position 7 on the

α-face of the *trans*-AB steroid nucleus, and therefore structurally related to our hydroborated cholesteryl polyamine conjugates, especially protected spermidine mimic **54** (Fig 86). Analogues of squalamine **101** have been designed, based on hydroxylated natural products, the bile acids, especially polyamine conjugates of lithocholic acid **17** (see also Chapter 2) or hyodeoxycholic acid **102** (Kikuchi *et al* 1997).

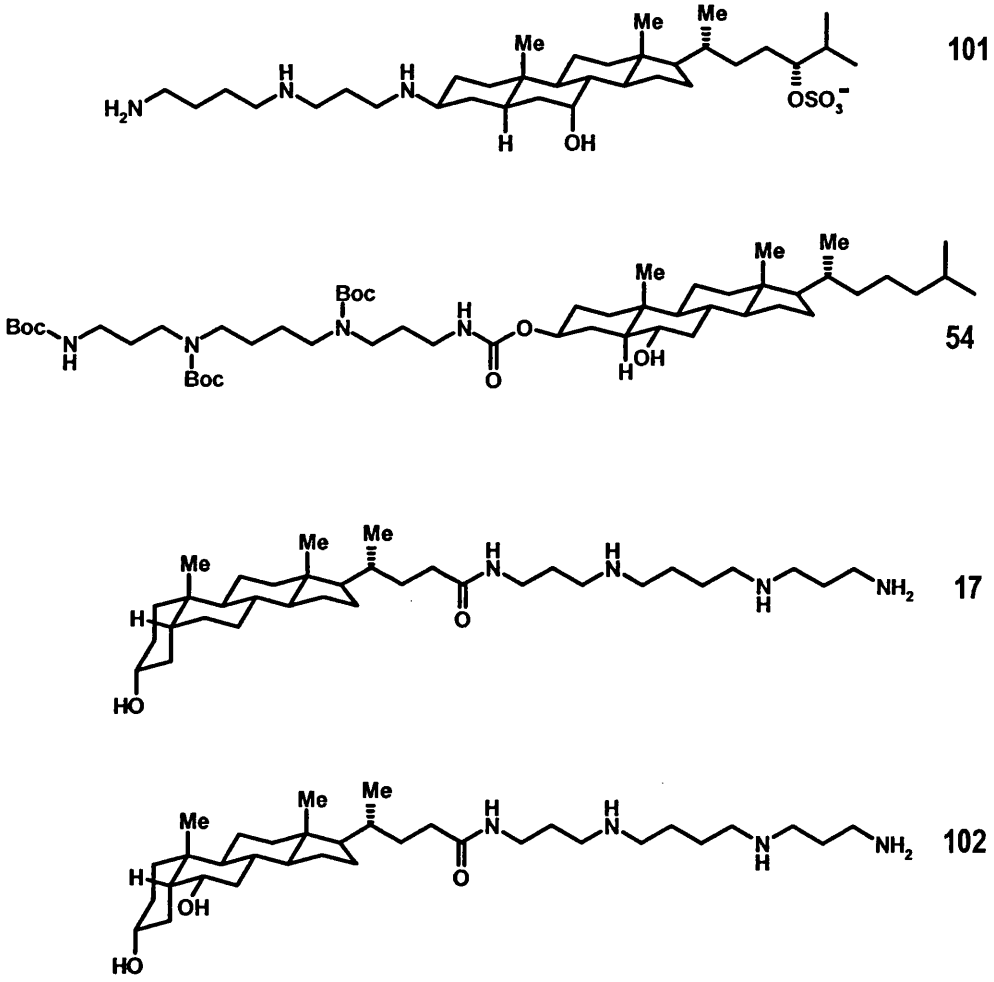


Figure 86. Structures of squalamine **101**, hydroborated cholesteryl carbamate **54**, lithocholic acid amide **17** and hyodeoxycholic acid amide **102**

Squalamine **101** and its analogues are currently in development as new antibiotics with an as yet undetermined mode of action and are therefore of clinical interest for use against resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA,

Kikuchi *et al* 1997). Lipopolyamine carbamates **NP-1**, **NP-2**, **NP-3**, **NP-4** and urea **NP-5** (Fig. 87) have been shown to be more potent against *S. aureus* than squalamine **101** *in vitro* (Kim *et al* 2001). The polyamine carbamates were synthesised by activation of the relevant alcohol with phosgene (20 % solution in toluene) to afford the chloroformate intermediate, in an analogous way to our synthesis of ergosteryl carbamates **30** and **33** (Kim *et al* 2001).

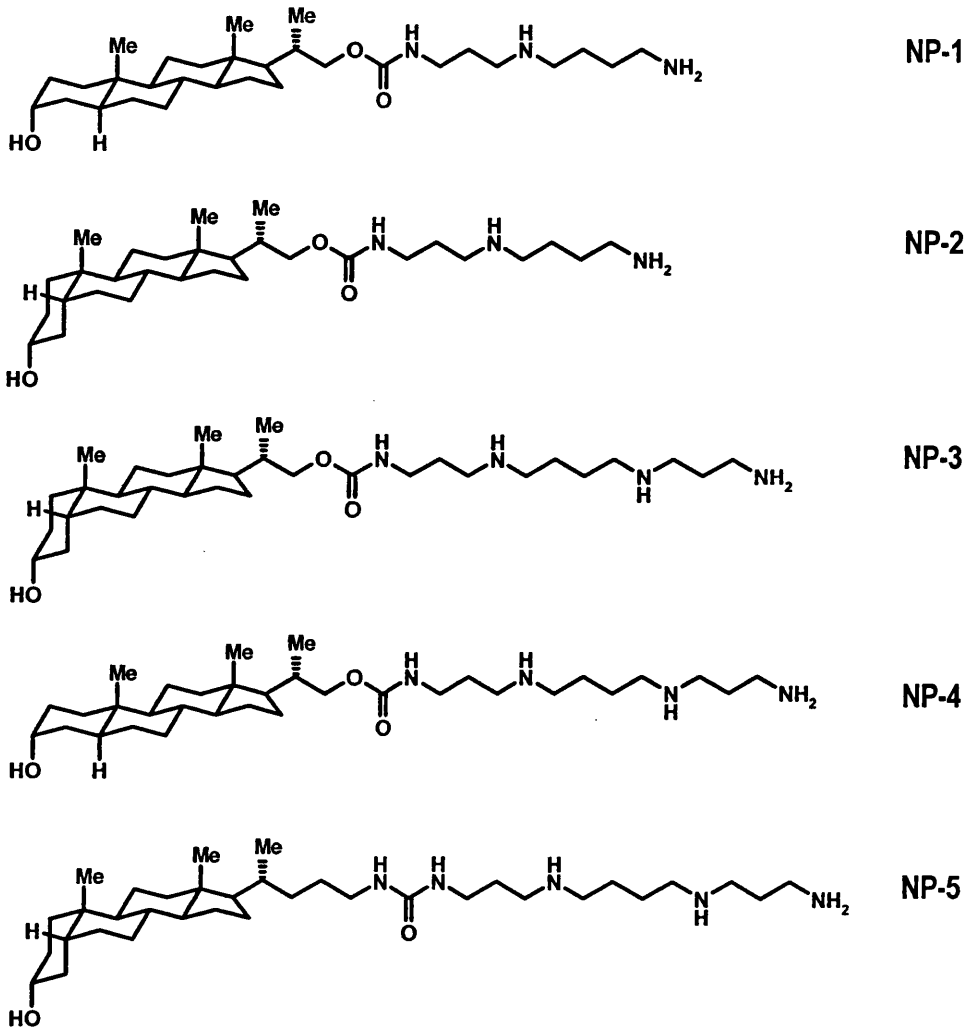


Figure 87. Structures of the Kim lipopolyamines **NP-1**, **NP-2**, **NP-3**, **NP-4** and **NP-5**

Lipopolyamines **NP-1**, **NP-2**, **NP-3**, **NP-4** and **NP-5** (Fig. 87) are relevant to our DNA condensation studies in that they vary in polyammonium moiety, steroid geometry and incorporate a carbamate or urea linker moiety. As part of our synthetic studies of *cis*- and

trans-AB ring steroids for the condensation of DNA, and in a collaboration with Professor Hong-Seok Kim (Kyungpook National University, South Korea), we were generously supplied with these steroidal polyamines.

Fig. 88 shows the EtBr displacement profiles of the five **NP**-lipopolyamines, with calf thymus DNA, at low salt concentration. As may be predicted from the literature (Geall *et al* 1998a, Blagbrough *et al* 2000) regarding hydroxylated steroids binding to DNA, spermidine mimics **NP-4** and **NP-5** condense calf thymus DNA with the greatest efficiency compared to putrescine mimics **NP-1** and **NP-2**. Also, the trends in binding between comparable *cis*- and *trans*-AB ring steroids show that the relative binding affinity of *cis*-AB lipopolyamines may be predicted from the observation that lithocholic acid polyamine amides (*cis*-AB) are less efficient in the condensation of DNA than their cholesterol-based counterparts. The *trans*-AB steroids **NP-4** and **NP-2** are more efficient than their *cis*-AB counterparts, **NP-1** and **NP-3** respectively. These observations may be explained by differences in the hydrophobic packing of bound steroid on the DNA (Muller *et al* 1995). In that case, perhaps surprisingly, **NP-5**, a *cis*-AB spermidine mimic with a urea linker has a similar EtBr displacement profile to that of **NP-4**. The alkyl urea linker in **NP-5** is two atoms longer than the corresponding carbamates of the other conjugates, and the linker is also a candidate for SAR studies in the design of new lipopolyamine conjugates for DNA condensation and potentially for non-viral gene delivery (Lee *et al* 1996).

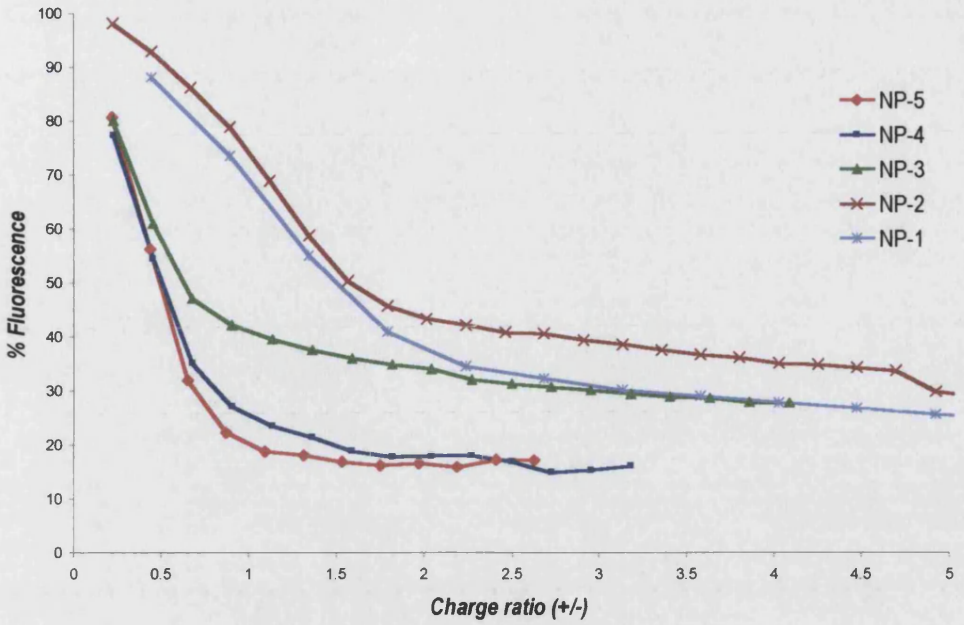


Figure 88. EtBr displacement profiles of lipopolyamines **NP-1**, **NP-2**, **NP-3**, **NP-4** and **NP-5**.

(Calf thymus DNA, 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA)

Naphthalimide-labelled lipopolyamines: DNA interactions studied by normal absorption

Fluorescent naphthalimide-conjugated lipopolyamines **93** and **94** (Fig. 89) were shown to be unsuitable for the EtBr displacement assay due to emission interference. Naphthalimide **93** absorbs at 260 nm and 450 nm and the emission band (531 nm) significantly overlaps with EtBr (600 nm) at increasing concentrations of naphthalimide (data not shown). Therefore, normal absorption spectra of calf thymus DNA-naphthalimide complexes at increasing charge ratio were measured (Fig. 90 and 91).

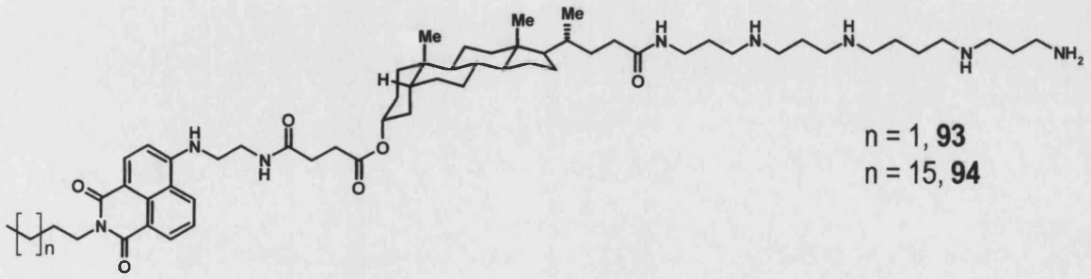


Figure 89. Structures of naphthalimide-lithocholic acid polyamine amides **93** and **94**

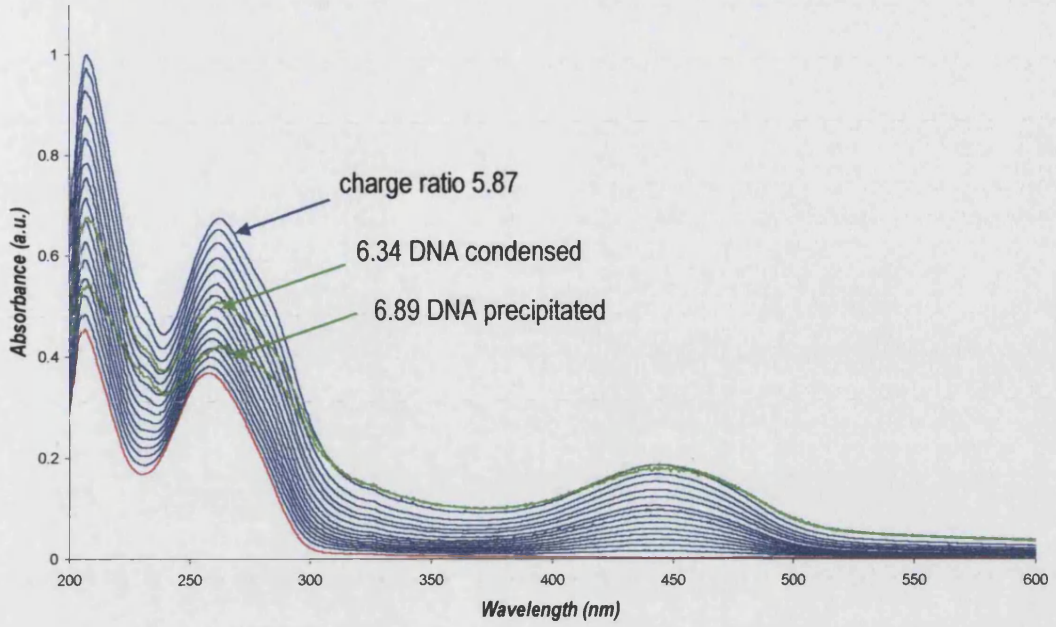


Figure 90. Normal absorption spectra of 60 μg calf thymus DNA (red) and 60 μg calf thymus DNA + increasing concentrations of naphthalimide conjugate **93** (blue): low salt 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA

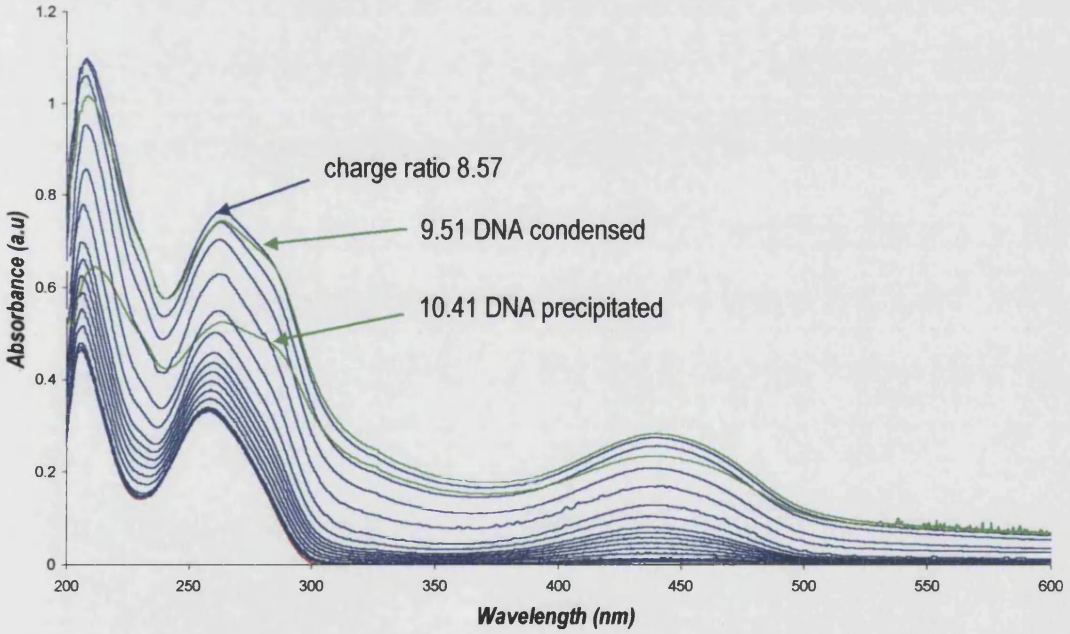


Figure 91. Normal absorption spectra of 60 μg calf thymus DNA (red) and 60 μg calf thymus DNA + increasing concentrations of naphthalimide conjugate **94** (blue): low salt 20 mM NaCl buffer, 2mM HEPES, 0.05 mM EDTA

The UV spectra (Fig. 90 and 91) show changes characteristic of polyamine binding and DNA condensation. Both conjugates cause the background light-scattering that accompanies DNA particle formation. Naphthalimide **93** causes the red shift and increased intensity of the band at 260 nm associated with the disruption of DNA-base stacking. Initially (charge ratio 0.28), naphthalimide **94** causes a lowering of the band at 260 nm, then further addition has the characteristic hyperchromic effect and light scattering is observed. Both naphthalimides **93** and **94** condense DNA upon addition of sufficient charge equivalents (6.34 for **93**, 9.51 for **94**). In both experiments, the appearance of the cuvette solution turned from yellow to colourless, as condensed DNA aggregated and was visible to the naked eye as a yellow precipitate. In addition to the observed turbidity, the DNA absorbance diminished across the spectrum. That the designed fluorescent lipopolyamines are visibly incorporated into DNA particles is an encouraging result for future work, where a fluorescent DNA particle may be useful in the imaging of transfection processes.

Naphthalimide conjugates **93** and **94** are spermine equivalents with 3.4 positive charges at physiological pH (Geall *et al* 2000). These vectors are more efficient than spermine (3.8 positive charges) in the condensation of calf thymus DNA. Naphthalimide **94** has a C18 lipid chain. Therefore, steric hindrance of ligand binding may be responsible for less efficient DNA condensation processes than observed with the n-butyl derivative **93**. Naphthalimides are known to be DNA intercalators (Tao *et al* 1996, Chang *et al* 1999). However, the absorbance for the naphthalimide band at 530 nm displayed linear hyperchromicity as a function of increased drug load, and no evidence for intercalation of this chromophore was observed. Naphthalimides **93** and **94** are substituted with bulky steroid and alkyl chains. These groups, especially the long octadecyl chain in conjugate **94**, may perturb insertion of the naphthalimide moiety into intercalation sites.

Conclusions

The synthesis of steroid based lipopolyamines has been investigated, with a design brief set in the area of efficient DNA condensation and non-viral gene delivery. We report the synthesis of spermidine and spermine polyammonium conjugates of cholesterol, lithocholic acid and ergosterol, a novel lipid moiety in the area of non-viral gene delivery. Using protecting-group strategies established in this research group and further refined here, spermine has been acylated, under regiochemical control, with the naturally occurring steroids cholesterol, lithocholic acid and ergosterol. These steroids afforded alkyl spermidine conjugates with variation in shape and hydrophobicity. In order to generate spermine-based polyammonium moieties for incorporation into lipopolyamines, spermine has been alkylated using a reductive alkylation route to extend unsymmetrically a polyamine chain. Also, the reduction of lithocholic acid amides to amines, as a route for retaining the pK_a of spermine moieties, has been successfully applied.

Using steroidal cationic lipids as analytical tools for the study of DNA condensation, we have investigated particle formation by studying the light scattering effect and also by EtBr displacement fluorescence assay. By applying such spectroscopic techniques, cholesteryl spermidine mimic **7** and poly-L-lysine (PLL) have been show to bind preferentially to A-T rich DNA sequences. PLL of various average molecular weights have been shown to condense DNA with different affinities (a 120-mer being more efficient than the 38-mer and 255-mer PLL), but generally are all suitable for efficient DNA condensation at physiological salt concentration (150 mM NaCl).

A synthetic monocationic ergosteryl carbamate, a model for DC-Chol, the first steroidal cationic lipid, has been synthesised and shown to condense calf thymus DNA with a greater efficiency than simple polyamines such as spermine. Also, lipoputrescine and lipospermidine

conjugates of other steroids have been assayed for their condensation of calf thymus DNA. Lipids possessing *trans*-AB ring junctions were more efficient than their *cis*-AB counterparts.

A synthetic route to fluorescent lipopolyamines has been established, via key Fmoc protected intermediates based on *cis*- and *trans*-AB steroid moieties. Using fluoride reagents to deprotect the Fmoc protecting group, we were able to introduce regioselectively fluorophores of choice to cholesterol and lithocholic based lipopolyamines. A crucial step in these achievements was the hydroboration of the alkene in cholesterol conjugates, in the presence of up to five carbamate functional groups. The major product was the *trans*-AB steroid, and it was separable from the minor product, the *cis*-AB diastereoisomer. Oregon Green and dansyl conjugates of cholesteryl spermine mimics have been prepared, each with the potential to be used together or independently in multicolour fluorescence studies related to DNA condensation and gene delivery.

Two naphthalimide-labelled conjugates of lithocholic acid-spermine mimics have been prepared. One incorporated both a C18 alkyl chain and a steroid moiety. Both conjugates were shown by normal absorption spectroscopy to condense calf thymus DNA and to be incorporated into a fluorescent DNA-lipopolyamine particle. We have designed, prepared, purified and analysed a series of complex small molecules, lipopolyamines that are capable of condensing DNA. We have made some of these efficient DNA-condensing agents fluoresce, creating the start of a series of useful molecular tools with potential in non-viral gene delivery.

CHAPTER 5

Experimental

General Experimental Details

Chemicals were purchased from Sigma-Aldrich-Fluka with the exception of fluorescein isothiocyanate (isomer I) and Oregon Green 488 NSE, which were purchased from Molecular Probes (Eugene OR, U.S.A.). Naphthalimide fluorophores were kindly supplied by Professor David Lewis (University of Wisconsin-Eau Claire WI, U.S.A.). Lipopolyamines **NP-1**, **NP-2**, **NP-3**, **NP-4** and **NP-5** were kindly supplied by Professor Hong-Seok Kim (Kyungpook National University, South Korea). All chemicals were used without further purification. Solvents were GPR or HPLC grade and purchased from BDH or Fisher. Anhydrous CH_2Cl_2 was distilled from calcium hydride and anhydrous MeOH was distilled from magnesium and iodine. Analytical thin layer chromatography (TLC) was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 F_{254}). Chromatograms were visualised with either UV light (254 or 366 nm), or stained with ninhydrin (0.3 % w/v solution in *n*-butanol-acetic acid 97:3 v/v), phosphomolybdic acid (5 % w/v solution in ethanol) or 2,4-dinitrophenylhydrazine (3.5 % w/v solution in ethanol-water-sulfuric acid 10:4:3 v/v/v) as appropriate. "Purified over silica gel" refers to preparative column chromatography with slurry packed silica gel 60 (35-75 μm) purchased from BDH, performed either under gravity or under pressure with hand bellows (flash chromatography) (Stille *et al* 1978). Sand (acid washed), purchased from BDH was used to seal the column of silica gel and was used after washing with acetone, ethyl acetate, methanol and CH_2Cl_2/MeOH/conc. aq. NH_3 (100:10:1 v/v/v). Solvents were "evaporated *in vacuo*" using a Büchi rotary evaporator under partial vacuum with a water pump.

Infra-red spectra were recorded as a KBr disc unless otherwise stated using a Perkin Elmer Spectrum RXI FT-IR instrument. ^1H NMR and ^{13}C NMR spectra were recorded on a JEOL EX400 instrument at 400 MHz and 100.8 MHz respectively. Chemical shifts (δ, in parts per million) are relative to tetramethylsilane in ^1H NMR spectra and solvent resonance in ^{13}C NMR spectra. Coupling constants (line separations) (*J*) are expressed in Hertz as absolute

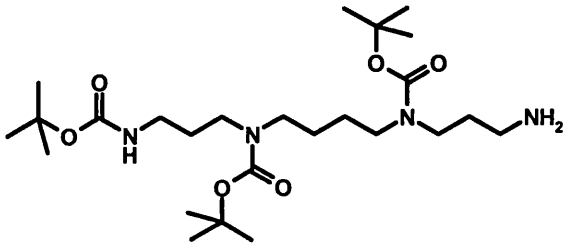
values and multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet) and b (broad). High and low resolution FAB mass spectra (positive ion mode, unless otherwise stated) were measured on a VG AutoSpec Q spectrometer, using *meta*-nitrobenzyl alcohol as the matrix. Polyamines are hygroscopic with varying salt content, therefore elemental analysis and melting points are unsuitable means of assessing the purity of these compounds (Byk *et al* 1997, Byk *et al* 1998). Semi-preparative RP-HPLC was performed with a Jasco PU-980 pump with a Jasco UV-975 detector. The stationary phase was a Supelcosil ABZ+Plus, 5 μm (25 cm x 10 mm) column. UV spectra were recorded on Perkin Elmer Lambda 40 or Unicam Helios γ spectrometers. Fluorescence measurements were made on a Perkin Elmer LS 50B fluorimeter.

Ethidium bromide displacement assay

DNA (calf thymus, poly $[d(A\text{-}T)]_2$, or poly $[d(G\text{-}C)]_2$, 6 μl, 1 mg/ml) in MilliQ water was added to buffer (20 mM or 150 mM NaCl, 2 mM HEPES, 0.05 mM EDTA, pH 7.4, 3000 μl) in a cuvette (quartz, 1 cm path length) followed by the addition of ethidium bromide (3 μl, 0.5 mg/ml). This solution was mixed (using a microflea) for 1 min and fluorescence intensity measured (λ_{ex} = 260 nm, λ_{em} = 600 nm). Aliquots of ligand (5 μl, typically 1 mg/ml stock solutions of polyamines, 0.1 mg/ml solutions of cationic polymers) were added with mixing, allowing 1 min equilibration time before measuring fluorescence. Curves were plotted as a function of ligand/DNA charge ratio, that is the ammonium/phosphate ion (N/P) ratio (Felgner *et al* 1997).

Light scattering assay (A_{320})

DNA (calf thymus, poly $[d(A\text{-}T)]_2$, or poly $[d(G\text{-}C)]_2$, 6 μl, 1 mg/ml) in MilliQ water was added with mixing to buffer (20 mM or 150 mM NaCl, 2 mM HEPES, 0.05 mM EDTA, pH 7.4, 3000 μl) in a cuvette (quartz, 1 cm path length). Absorbance at λ = 320 nm was measured. Aliquots of ligand (5 μl, typically 1 mg/ml solutions of cationic polymers) were added with mixing, allowing 1 min equilibration time before measuring the absorbance. Curves were plotted as a function of ligand/DNA charge ratio.



***N*^{1},*N*^{4},*N*^{9}-(Tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 5**

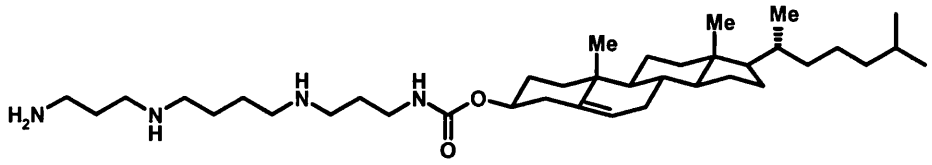
A solution of ethyl trifluoroacetate (3.09 g, 21.7 mmol) in MeOH (50 ml) was added dropwise over 30 min to a stirred solution of 1,12-diamino-4,9-diazadodecane **1** (4.00 g, 19.8 mmol) in MeOH (300 ml) at -78 °C, under nitrogen. The temperature was increased to 0 °C over 1 h, then di-*tert*-butyl dicarbonate (17.23 g, 79 mmol) in MeOH (50 ml) was added dropwise over 5 min. The reaction mixture was allowed to warm to 25 °C, then stirred for a further 18 h. After this time, the solution was basified (pH > 11) with conc. aq. ammonia and stirred for a further 18 h. The solution was then concentrated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 75:10:1 v/v/v) to afford the title compound as a colourless oil (4.86 g, 49 %). R_f 0.20 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR ($CDCl_3$): 1.43-1.48 (31 H, m, 6-CH_2, 7-CH_2, O-C($C\underline{H}_3$)$_3$ x 3), 1.60-1.66 (2 H, m, 2-CH_2), 1.74-1.80 (2 H, m, 11-CH_2), 2.72-2.81 (2 H, m, 12-CH_2), 3.05-3.32 (10 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2),

4.12 (2 H, s, NH_2). ^{13}C NMR: 25.9 (6-CH_2, 7-CH_2), 28.3, 28.4, 28.4 (2-CH_2, O-C($\underline{C}H_3$)$_3$ x 3, overlapping), 37.5 (1-CH_2), 38.9 (12-CH_2), 43.9, 44.3 (3-CH_2, 10-CH_2), 46.8 (5-CH_2, 8-CH_2, overlapping), 78.8, 79.4 (O-$\underline{C}$(CH_3)$_3$ x 3, overlapping), 155.7 (N-$\underline{C}$O-O-C(CH_3)$_3$ x 3). FAB-MS *m/z* 503 (MH^+), $C_{34}H_{56}N_2O_2$ requires 502; FAB-HRMS calculated 503.3809 (MH^+), found 503.3829.

N^1-(Cholesteryl-3'-oxycarbonyl)-N^4,N^9,N^{12}-(tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 6

Cholesteryl chloroformate (738 mg, 1.6 mmol) in CH_2Cl_2 (10 ml) was added over 5 min to a stirred solution of tri-Boc protected polyamine **5** (750 mg, 1.5 mmol) in CH_2Cl_2 (20 ml) with TEA (0.5 ml) at 25 °C, under nitrogen. The solution was stirred for 6 h then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 1:9 to 4:6 v/v) to afford the title compound as a white solid (1.17 g, 86 %). R_f 0.65 (EtOAc-hexane 7:3 v/v). ^1H NMR ($CDCl_3$): 0.68 (3 H, s, 18'-CH_3), 0.86, 0.87 (6 H, 2 x d, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.91 (3 H, d, J = 6, 21'-CH_3), 1.01 (3 H, s, 19'-CH_3), 1.04-2.02 (61 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 3), 2.27-2.36 (2 H, m, 24'-CH_2), 3.05-3.35 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 4.43-4.53 (1 H, m, 3'-CH), 5.37 (1 H ,m, 6'-CH), 5.60-5.65 (2 H, m, CH_2-N$\underline{H}$-CO x 2). ^{13}C NMR: 11.6 (18'-CH_3), 18.5 (21'-CH_3), 19.1 (19'-CH_3), 20.8 (11'-CH_2), 22.4 (27'-CH_3), 22.6 (26'-CH_2), 23.6 (23'-CH_2), 24.1 (15'-CH_2), 25.5, 25.8 (6-CH_2, 7-CH_2), 27.8, 28.0, 28.0, 28.2, 28.7 (2-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C($\underline{C}H_3$)$_3$ x 3, overlapping), 31.7 (7'-CH_2, 8'-CH), 35.6 (20'-CH), 36.0 (22'-CH_2), 36.3 (10'-C), 36.8 (1'-CH_2), 37.3 (1-CH_2, 12-CH_2), 38.4 (24'-CH_2), 39.3, 39.5 (4'-CH_2, 12'-CH_2), 42.1 (13'-C), 43.7, 44.0 (3-CH_2, 10-CH_2), 46.1, 46.6 (5-CH_2, 8-CH_2), 49.8 (9'-CH), 55.9 (17'-CH), 56.5 (14'-CH_2), 73.9 (3'-CH), 79.4 (O-$\underline{C}$(CH_3)$_3$ x 3), 122.1 (6'-CH), 139.8 (5'-C), 155.9 (N-$\underline{C}$O-O-

$C(CH_3)_3$ x 3, NH-CO-O-CH, overlapping). FAB-MS *m/z* 915 (MH^+), $C_{53}H_{94}N_4O_8$ requires 914; FAB-HRMS calculated 915.7150 (MH^+), found 915.7152.



N^1-(Cholesteryl-3'-oxycarbonyl)-1,12-diamino-4,9-diazadodecane tritrifluoroacetate salt 7

Tri-Boc protected polyamine **5** (215 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (18 ml) and TFA (2 ml) was stirred at 25 °C for 4 h, then the solvent was evaporated *in vacuo* to afford the title compound as a white solid (114 mg, 79 %). 10 mg of this material was purified by semi-preparative RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 220 nm, MeCN-0.1 % aq. TFA 6:4 v/v), t_R 7.7 min. ^1H NMR (d_6-DMSO): 0.65 (3 H, s, 18'-CH_3), 0.84 (6 H, d, *J* = 6, 26'-CH_3, 27'-CH_3), 0.90 (3 H, d, *J* = 6, 21'-CH_3), 0.97 (3 H, s, 19'-CH_3), 0.97-2.00 (35 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH), 2.16-2.32 (2 H, m, 24'-CH_2), 2.80-3.07 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 4.22-4.38 (1 H, m, 3'-CH), 5.26-5.38 (1 H, m, 6'-CH), 8.00-8.95 (bs, ammonium NH). ^{13}C NMR: 12.0 (18'-CH_3), 18.8 (21'-CH_3), 19.3 (19'-CH_3), 20.8 (11'-CH_2), 22.6 (26'-CH_3), 22.9, 22.9 (6-CH_2, 7-CH_2, 27'-CH_2), 23.5 (23'-CH_2), 23.9, 24.0 (2-CH_2, 15'-CH_2), 26.5 (11-CH_2), 27.7 (16'-CH_2), 31.6 (7'-CH_2, 8'-CH), 35.5 (20'-CH), 35.9 (22'-CH_2), 36.3 (10'-C), 36.4 (1'-CH_2), 36.8 (1-CH_2), 37.6 (12-CH_2), 38.5 (24'-CH_2), 39.1, 39.1 (4'-CH_2, 12'-CH_2), 42.1 (13'-C), 44.1 (3-CH_2), 44.8 (10-CH_2), 46.3, 46.5 (5-CH_2, 8-CH_2), 49.7 (9'-CH), 55.8 (17'-CH), 56.3 (14'-CH_2), 73.2 (3'-CH), 121.9 (6'-CH), 139.7 (5'-C), 155.8 (NH-CO-O-CH). FAB-MS *m/z* 615 (MH^+), $C_{38}H_{70}N_4O_2$ requires 614.

N^1,N^{12}-Ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane ditrifluoroacetate salt 8

Ethyl trifluoroacetate (7.19 g, 50.65 mmol) was added to a stirred solution of 1,12-diamino-4,9-diazadodecane **1** (2.05 g, 10.13 mmol) and water (0.36 ml, 20.26 mmol) in MeCN (45 ml) at 25 °C. The solution was heated at reflux for 18 h, then cooled to 25 °C and filtered to give a white solid. Recrystallisation from EtOAc afforded the title compound as a white solid (5.99 g, 93 %). IR 1684 cm^{-1} (CO). ^1H NMR (d_6-DMSO): 1.60-1.70 (4 H, m, 6-CH_2, 7-CH_2), 1.85 (4 H, quin, J = 7, 2-CH_2, 11-CH_2), 2.88-3.00 (8 H, m, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2), 3.27 (4 H, q, J = 6, 1-CH_2, 12-CH_2), 8.80-9.00 (4 H, m, NH_2^+ x 2), 9.66 (2 H, br t, J = 5, CF_3-CO-NH x 2). ^{13}C NMR: 22.8 (6-CH_2, 7-CH_2), 25.1 (2-CH_2, 11-CH_2), 36.7 (5-CH_2, 8-CH_2), 44.5 (3-CH_2, 10-CH_2), 46.1 (1-CH_2, 12-CH_2), 113.4-120.1 (m, $\underline{C}F_3CO$-NH x 2, $\underline{C}F_3CO$-O x 2, overlapping), 156.3 (q, $^2J_{CF}$ = 36, $CF_3\underline{C}O$-NH x 2), 158.8 (q, $^2J_{CF}$ = 31, $CF_3\underline{C}O$-O x 2). FAB-MS m/z 395 (MH^+), $C_{14}H_{24}N_4O_2F_6$ requires 394.

N^4-(Cholesteryl-3'-oxycarbonyl)-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane 9

A solution of ethyl trifluoroacetate (1.81 g, 10.6 mmol) in CH_2Cl_2 (20 ml) was added dropwise over 30 min to a stirred solution of 1,12-diamino-4,9-diazadodecane **1** (1.00 g, 4.9 mmol) in CH_2Cl_2 (30 ml) at -78 °C, under nitrogen. The temperature was increased to 0 °C over 1 h, then TEA (2.3 ml, 15.9 mmol) was added. Cholesteryl chloroformate (2.39 g, 5.3 mmol) in CH_2Cl_2 (25 ml) was added dropwise over 30 min. The reaction mixture was allowed to warm to 25 °C, then stirred for a further 18 h. The solvent was evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford the title compound as a white wax (3.03 g, 76 %). R_f 0.38 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR ($CDCl_3$): 0.68 (3 H, s, 18'-CH_3), 0.86, 0.87 (6 H, 2 x d, J = 7, overlapping 2

Hz, 26'-CH_3 and 27'-CH_3), 0.92 (3 H, d, J = 6, 21'-CH_3), 1.02 (3 H, s, 19'-CH_3), 1.04-2.03 (35 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH), 2.25-2.38 (2 H, m, 24'-CH_2), 3.17-3.50 (8 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2), 4.48-4.58 (1 H, m, 3'-CH), 5.37-5.41 (1 H, m, 6'-CH), 8.20-8.51 (bs, NH-CO). ^{13}C NMR: 11.9 (18'-CH_3), 18.7 (21'-CH_3), 19.3 (19'-CH_3), 21.0 (11'-CH_2), 22.6 (27'-CH_3), 22.8 (26'-CH_3), 23.8 (23'-CH_2), 24.3 (15'-CH_2), 26.2, 26.8, 27.1, 28.0, 28.2, 28.2 (2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH), 31.9 (8'-CH), 32.0 (7'-CH_2, 8-CH_2), 35.8 (20'-CH), 36.0 (5-CH_2), 36.2 (22'-CH_2), 36.6 (10'-C), 36.9 (1'-CH_2), 38.6 (24'-CH_2), 39.5, 39.7 (4'-CH_2, 12'-CH_2), 40.9 (3-CH_2), 42.3 (13'-C), 43.5 (10-CH_2), 46.7 (1-CH_2), 49.4 (12-CH_2), 50.0 (9'-CH), 56.1 (17'-CH), 56.7 (14'-CH_2), 75.3 (3'-CH), 116.0, 116.1 (2 x q, $^1J_{CF}$ = 289 , overlapping 13 Hz, $\underline{C}F_3$-CONH x 2), 122.8 (6'-CH), 139.5 (5'-C), 155.7-157.9 (m, CF_3-$\underline{C}$ONH x 2, NH-$\underline{C}$O-O-CH overlapping). FAB-MS *m/z* 807 (MH^+), $C_{42}H_{68}N_4O_4F_6$ requires 806; FAB-HRMS calculated 807.5223 (MH^+), found 807.5227.

N^4-(Cholesteryl-3'-oxycarbonyl)-N^1,N^8-ditrifluoroacetyl-1,8-diamino-4-azaoctcane 12

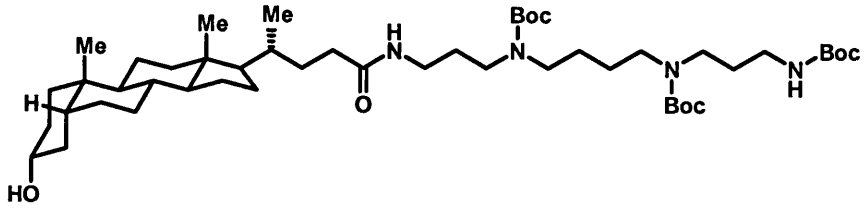
A solution of ethyl trifluoroacetate (525 mg, 3.7 mmol) in CH_2Cl_2 (10 ml) was added dropwise over 30 min to a stirred solution of 1,8-diamino-4-azaoctcane **2** (244 mg, 1.7 mmol) in CH_2Cl_2 (30 ml) at -78 °C, under nitrogen. The temperature was increased to 0 °C over 1 h, then TEA (0.7 ml, 5.1 mmol) was added. Cholesteryl chloroformate (829 mg, 1.8 mmol) in CH_2Cl_2 (15 ml) was added dropwise over 30 min. The reaction mixture was allowed to warm to 25 °C, then stirred for a further 18 h. The solvent was evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-hexane 4:1 to 100:1 v/v) to afford the title compound as a white powder (1.16 g, 92 %). R_f 0.68 (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 0.68 (3 H, s, 18'-CH_3), 0.86, 0.87 (6 H, 2 x d, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.92 (3 H, d, J = 6, 21'-CH_3), 1.02 (3 H, s, 19'-CH_3), 1.04-2.02 (33 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH,

9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH), 2.23-2.37 (2 H, m, 24'-CH_2), 3.19-3.44 (8 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2), 4.48-4.58 (1 H, m, 3'-CH), 5.38 (1 H, m, 6'-CH), 6.67, 8.13 (2 H, 2 x m, NH-CO x 2). ^{13}C NMR: 12.0 (18'-CH_3), 18.8 (21'-CH_3), 19.4 (19'-CH_3), 21.1 (11'-CH_2), 22.6 (27'-CH_3), 22.9 (26'-CH_2), 23.9 (23'-CH_2), 24.4 (15'-CH_2), 25.8, 25.8 (6-CH_2, 7-CH_2), 27.4, 27.5, 28.1, 28.3 (2-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH), 31.9, 32.0 (7'-CH_2, 8'-CH), 35.8 (20'-CH), 36.2 (22'-CH_2), 36.6 (10'-C), 37.0 (1'-CH_2), 38.6 (24'-CH_2), 39.2 (3-CH_2, 5-CH_2), 39.4, 39.6 (4'-CH_2, 12'-CH_2), 42.4 (13'-C), 43.8 (1-CH_2, 8-CH_2), 50.0 (9'-CH), 56.1 (17'-CH), 56.7 (14'-CH_2), 75.5 (3'-CH), 122.7 (6'-CH), 139.3 (5'-C), 157.0, 157.4 (CF_3-CONH x 2, NH-CO-O-CH overlapping). FAB-MS *m/z* 772 ($M+Na^+$), $C_{39}H_{61}N_3O_4F_6$ requires 749; FAB-MS *m/z* 748 $(M-H^+)^-$, FAB-HRMS calculated 748.4488 $(M-H^+)^-$, found 748.4508.

N^4-(*tert*-Butyloxycarbonyl)-N^4-(cholesteryl-3'-oxycarbonyl)-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane 14

To a stirred solution of triacylated polyamine **9** (478 mg, 0.59 mmol) in CH_2Cl_2 (10 ml), was added di-*tert*-butyl dicarbonate (142 mg, 0.65 mmol) and TEA (1 ml, 7.7 mmol) in CH_2Cl_2 (2 ml) at 25 °C. The solution was stirred for 1 h then evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-hexane 1:1 to 100:1 v/v) to afford the title compound as a white foam (329 mg, 64 %). R_f 0.46 (CH_2Cl_2-hexane 7:3 v/v). ^1H NMR ($CDCl_3$): 0.68 (3 H, s, 18'-CH_3), 0.86, 0.87 (6 H, 2 x d, J = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.92 (3 H, d, J = 6, 21'-CH_3), 1.02 (3 H, s, 19'-CH_3), 1.03-2.05 (44 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH, O-C(CH_3)$_3$), 2.23-2.38 (2 H, m, 24'-CH_2), 3.20-3.40 (8 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2), 4.48-4.59 (1 H, m, 3'-CH), 5.35-5.40 (1 H, m, 6'-CH), 8.10-8.38 (bs, NH-CO). ^{13}C NMR: 12.0 (18'-CH_3), 18.8 (21'-CH_3), 19.4 (19'-CH_3), 21.1 (11'-CH_2), 22.7 (27'-

CH_3), 22.9 (26'-CH_3), 23.9 (23'-CH_2), 24.4 (15'-CH_2), 26.2, 26.3, 27.5, 27.6 (2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2,), 28.4 (25'-CH), 28.6 (2'-CH_2, 16'-CH_2), 28.8 (O-C($\underline{C}H_3$)$_3$), 31.9 (8'-CH), 32.0 (7'-CH_2, 8-CH_2), 35.9 (20'-CH), 36.0 (5-CH_2), 36.2 (22'-CH_2), 36.6 (10'-C), 37.0 (1'-CH_2), 38.7 (24'-CH_2), 39.6, 39.8 (4'-CH_2, 12'-CH_2), 42.4 (13'-C), 43.6, 43.6 (3-CH_2, 10-CH_2), 46.7, 46.8 (1-CH_2, 12-CH_2), 50.0 (9'-CH), 56.1 (17'-CH), 56.7 (14'-CH_2), 75.4 (3'-CH), 80.9 (O-$\underline{C}$(CH_3)$_3$), 122.8 (6'-CH), 139.2 (5'-C), 157.2, 157.6 (CF_3-$\underline{C}$ONH x 2, O-C($\underline{C}H_3$)$_3$, NH-$\underline{C}$O-O-CH overlapping).



N^4,N^9,N^{12}-(Tri-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,12-diamino-4,9-diazadodecane 16

EDC (328 mg, 1.71 mmol) and lithocholic acid (321 mg, 0.85 mmol) were dissolved in CH_2Cl_2 (5 ml) and HOBt (58 mg, 0.43 mmol) was added with stirring at 25 °C under nitrogen. Tri-Boc protected polyamine **5** (428 mg, 0.85 mmol) was added and the solution stirred for 16 h. CH_2Cl_2 (30 ml) was added and the solution was washed with water (50 ml) and saturated $NaHCO_3$ solution (60 ml). The organic layers were dried ($MgSO_4$), evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 30:1 to 20:1 v/v) to afford the title compound as a white foam (700 mg, 95%). R_f 0.24 (CH_2Cl_2-MeOH 20:1 v/v). ^{1}H NMR ($CDCl_3$): 0.61 (3 H, s, 18'-CH_3), 0.89-2.29 (69 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 19'-CH_3, 20'-CH, 21'-CH_3, 22'-CH_2, 23'-CH_2, O-C(CH_3)$_3$ x 3), 3.02-3.34 (12 H, m, 1-

CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 3.55-3.64 (1 H, m, 3'-CH), 5.27-5.36 (1 H, m, NH-CO-O), 6.71-6.86 (1 H, bs, 1-CH_2-N<u>H</u>-CO). ^{13}C NMR: 12.5 (18'-CH_3), 18.8 (21'-CH_3), 21.2 (11'-CH_2), 23.8 (19'-CH_3), 24.6 (15'-CH_2), 25.5, 26.2 (6-CH_2, 7-CH_2), 26.8 (7'-CH_2), 27.6 (6'-CH_2), 27.6, 27.6 (2-CH_2, 11-CH_2), 28.6 (16'-CH_2), 28.8 (O-C(<u>C</u>H_3)$_3$ x 3), 30.9 (2'-CH_2), 32.2 (22'-CH_2), 34.9 (10'-C, 23'-CH_2, overlapping), 35.8 (1'-CH_2), 35.9 (20'-CH), 36.2 (8'-CH), 36.8 (4'-CH_2) 37.4, 37.4 (1-CH_2, 12-CH_2), 40.6 (12'-CH_2), 40.8 (9'-CH), 42.5 (5'-CH), 43.1 (13'-C), 44.4, 44.8, 46.4, 46.6, 46.9 (3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2), 56.4 (17'-CH), 56.8 (14'-CH), 72.1 (3'-CH), 80.0 (O-<u>C</u>$(CH_3)_3$ x 3), 156.2 156.4 (O-CO-NH x 3), 174.0 (NH-CO). FAB-MS *m/z* 862 (MH^+), $C_{49}H_{88}N_4O_8$ requires 862; FAB-HRMS calculated 861.6680 (MH^+), found 861.6667.

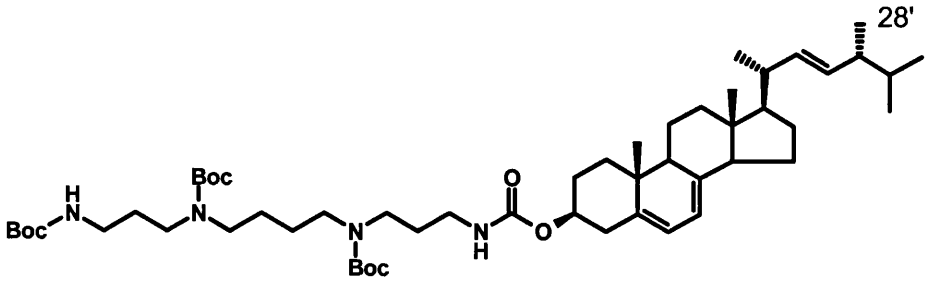
N^1-(3'α-Trifluoroacetoxy-5'β-cholan-24'-carbonyl)-1,12-diamino-4,9-diazadodecane tritrifluoroacetate salt 18 (Attempted synthesis of N^1-(3α-Hydroxy-5β-cholan-24-carbonyl)-1,12-diamino-4,9-diazadodecane trifluoroacetate salt **17**)

Tri-Boc protected polyamine **16** (500 mg, 0.58 mmol) in anhydrous CH_2Cl_2 (18 ml) and TFA (2 ml) was stirred at 0 °C for 4 h, then the solvent was evaporated *in vacuo*. The residue was washed with diethyl ether (3 x 10 ml) then recrystallised from EtOAc to afford the title compound as a white solid (266 mg, 70 %). ^1H NMR (d_6-DMSO): 0.60 (3 H, s, 18'-CH_3), 0.86 (3 H, d, *J* = 6, 21'-CH_3), 0.90 (3 H, s, 19'-CH_3), 0.92-2.12 (35 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2), 2.80-3.02 (10 H, m, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 3.08 (2 H, q, *J* = 6, 1-CH_2), 4.87-4.98 (1 H, m, 3'-CH), 7.29 (1:1:1, t, $^1J_{NH}$ = 52, NH), 8.04, 8.77, 8.94 (7 H, 3 x bs, ammonium NH). ^{13}C NMR: 12.1 (18'-CH_3), 18.5 (21'-CH_3), 20.7 (11'-CH_2), 22.9, 23.0 (6-CH_2, 7-CH_2), 23.1 (19'-CH_3), 24.6 (2-CH_2, 15'-CH_2), 26.1, 26.3 (11-CH_2, 7'-CH_2), 26.7 (6'-CH_2), 28.0 (16'-CH_2), 31.4 (2'-CH_2), 31.8 (22'-CH_2), 32.6 (23'-CH_2), 34.3 (10'-C), 35.3 (1'-CH_2, 20'-CH), 35.5 (8'-CH), 35.8 (1-CH_2) 36.4 (4'-CH_2, 12-CH_2),

39.7 (12'-CH_2), 40.1 (9'-CH), 41.3 (5'-CH), 42.5 (13'-C), 44.1, 44.9, 46.3 (3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2 overlapping), 55.8 (17'-CH), 56.0 (14'-CH), 79.5 (3'-CH), 113.5-119.0 (m, $\underline{C}F_3CO$-O-CH, $\underline{C}F_3CO$-O x 3, overlapping), 156.0-159.9 (m, $CF_3\underline{C}O$-O-CH, $CF_3\underline{C}O$-O x 3, overlapping), 173.0 (NH-CO). FAB-MS *m/z* 657 (MH^+), $C_{36}H_{63}N_4O_3F_3$ requires 656; FAB-HRMS calculated 657.4931 (MH^+), found 657.4961.

6-Benzyloxycarbonylaminohexanyl-1-oxycarbonylpropylamine 28

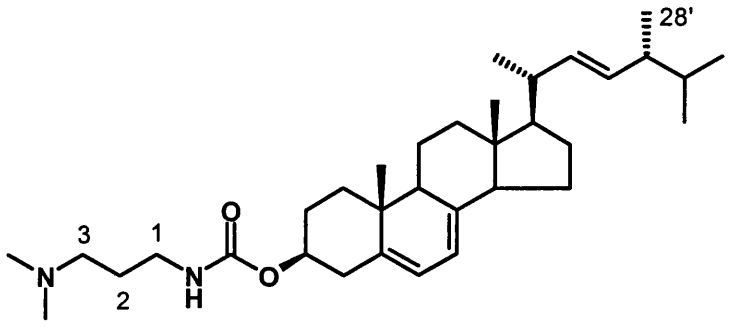
To a stirred solution of 6-benzyloxycarbonylaminohexan-1-ol **41** (1.05 g, 4.18 mmol) and 1,1-carbonyldiimidazole (1.02 g, 6.27 mmol) in CH_2Cl_2 (20 ml) was added TEA (1 ml). The solution was heated to reflux for 2 h, then cooled to 25 °C. 1-Propylamine (493 mg, 8.36 mmol), was added and the solution stirred for 2 h, then evaporated to dryness *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 300:10:1 v/v/v) to afford the title compound as a white solid (900 mg, 64 %). R_f 0.45 (CH_2Cl_2-MeOH-conc. aq. NH_3 200:10:1 v/v/v). IR 1684 cm^{-1} (N-CO). ^1H NMR ($CDCl_3$): 0.93 (3 H, t, *J* = 7, CH_2-$C\underline{H}_3$), 1.28-1.40 (4 H, m, 3-CH_2, 4-CH_2), 1.42-1.54 (6 H, m, 2-CH_2, 5-CH_2, $C\underline{H}_2$-CH_3), 3.12, 3.18 (4 H, 2 x q, *J* = 7, overlapping 23 Hz, 6-CH_2, N-$C\underline{H}_2$-CH_2-CH_3), 4.03 (2 H, t, *J* = 7, 1-CH_2), 4.87-4.94 (1 H, bs, 6-CH_2-N$\underline{H}$-CO-O), 4.76-4.83 (1 H, bs, CH_3-CH_2-CH_2-N$\underline{H}$-CO-O), 5.09 (2 H, s, O-CH_2-Ph), 7.28-7.38 (5 H, m, Ph). ^{13}C NMR: 11.3 (3'-CH_3), 23.2 ($\underline{C}H_2$-CH_3), 25.5 (3-CH_2), 26.3 (4-CH_2),28.9 (2-CH_2), 29.8 (5-CH_2), 40.9 (6-CH_2), 42.7 (CH_3-CH_2-$\underline{C}H_2$), 64.5 (1-CH_2), 66.5 (O-CH_2-Ph), 127.9, 128.3 (Ph), 136.4 (C Ph), 156.2, 156.5 (6-CH_2-NH-$\underline{C}$O-O, CH_3-CH_2-CH_2-NH-$\underline{C}$O-O). FAB-MS *m/z* 337, (MH^+), $C_{18}H_{28}N_2O_4$ requires 336; FAB-HRMS calculated 337.2127 (MH^+), found 337.2131.



N^1-(Ergosteryl-3'-oxycarbonyl)-N^4,N^9,N^{12}-(tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 30

Phosgene (20 % solution in toluene, 10 ml, 20.4 mmol) was added dropwise to a stirred solution of ergosterol (1.37 g, 3.60 mmol) in THF (10 ml) at 25 °C. This solution was stirred for 2 h then evaporated to dryness *in vacuo*. The residue was redissolved in anhydrous CH_2Cl_2 (5 ml) and added dropwise to a stirred solution of tri-Boc protected polyamine **5** (1.65 g, 3.29 mmol) and TEA (1 ml 7.7 mmol) in anhydrous CH_2Cl_2 (10 ml) and then stirred for 16 h. The solution was evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 3:7 to 7:3 v/v) to afford the title compound as a yellow foam (1.93 g, 60 %). R_f 0.62 (EtOAc-hexane 7:3 v/v). IR 1694 cm^{-1} (N-CO-O). ^1H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.82, 0.84 (6 H, 2 x d, J = 7, 26'-CH_3, 27'-CH_3), 0.92 (3 H, d, J = 7, 28'-CH_3), 0.94 (3 H, s, 19'-CH_3), 1.04 (3 H, d, J = 7, 21'-CH_3), 1.20-2.57 (55 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 24'-CH, 25'-CH, O-C(CH_3)$_3$ x 3), 3.02-3.34 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 4.52-4.64 (1 H, m, 3'-CH), 5.13-5.26 (2 H, m, 22'-CH, 23'-CH), 5.34-5.40 (1 H, m, 7'-CH), 5.53-5.58 (1 H, m, 6'-CH). ^{13}C NMR: 12.5 (18'-CH_3), 16.6 (19'-CH_3), 18.0 (28'-CH_3), 20.0, 20.4 (26'-CH_3, 27'-CH_3), 21.4 (11'-CH_2), 21.5 (21'-CH_3), 23.4 (15'-CH_2), 25.8, 26.4 (6-CH_2, 7-CH_2), 28.7, 28.8, 28.9 (2-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, O-C($\underline{C}H_3$)$_3$ x 3), 33.4 (25'-CH), 37.4 (10'-C), 37.5 (4'-CH_2), 37.7 (1-CH_2, 12-CH_2, overlapping), 38.3 (1'-CH_2), 39.4 (12'-CH_2), 40.8 (20'-CH), 43.1

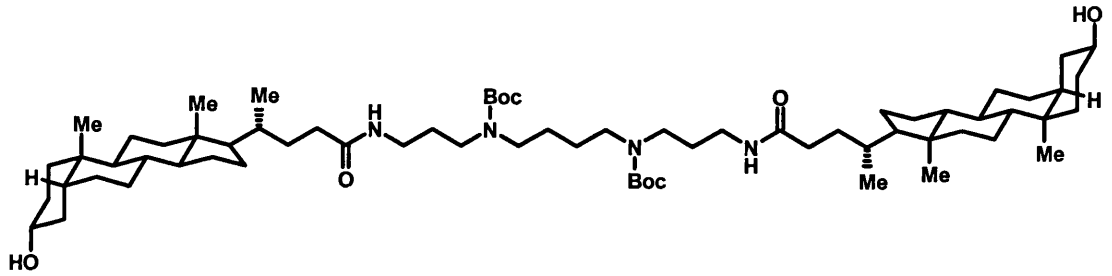
(13'-C, 24'-CH overlapping), 44.1, 44.5 (3-CH_2, 10-CH_2), 46.4 (9'-CH), 46.6 (5-CH_2, 8-CH_2, overlapping), 54.8 (14'-CH), 56.0 (17'-CH), 73.1 (3'-CH), 79.6 (O-C$(CH_3)_3$ x 3), 116.5 (7'-CH), 119.6 (6'-CH), 132.0 (23'-CH), 135.7 (22'-CH), 140.0 (8'-C), 141.5 (5'-C), 156.2 (N-CO-O). FAB-MS *m/z* 925 (MH^+), $C_{54}H_{92}N_4O_8$ requires 924; FAB-HRMS calculated 925.6993 (MH^+), found 925.6987.



1-(Ergosteryl-3'-oxycarbonylamino)-3-*N,N*-dimethylaminopropane 33

Phosgene (20 % solution in toluene, 5 ml, 10.2 mmol) was added dropwise to a stirred solution of ergosterol (800 mg, 2.02 mmol) in THF (10 ml) at 25 °C. This solution was stirred for 2 h then evaporated to dryness *in vacuo*. The residue was redissolved in anhydrous CH_2Cl_2 (5 ml) and added dropwise to a stirred solution of *N,N*-dimethylamino-3-propylamine (413 mg, 4.04 mmol) in anhydrous CH_2Cl_2 (10 ml) and then stirred for 1 h. The solution was evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 75:10:1 v/v/v) to afford the title compound as a buff solid (889 mg, 84 %). R_f 0.24 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.82, 0.84 (6 H, 2 x d, J = 7, 26'-CH_3, 27'-CH_3), 0.92 (3 H, d, J = 7, 28'-CH_3), 0.94 (3 H, s, 19'-CH_3), 1.04 (3 H, d, J = 7, 21'-CH_3), 1.21-2.56 (30 H, m, 1'-CH_2, 2'-CH_2, 4'-CH_2, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 24'-CH, 25'-CH, $(CH_3)_2N$, 2-CH_2, 3-CH_2), 3.22-3.26 (2 H, m, 1-CH_2), 4.56-4.61 (1 H, m, 3'-CH), 5.14-5.25 (2 H, m, 22'-CH, 23'-CH), 5.37-5.38 (1 H, m, 7'-CH), 5.53-

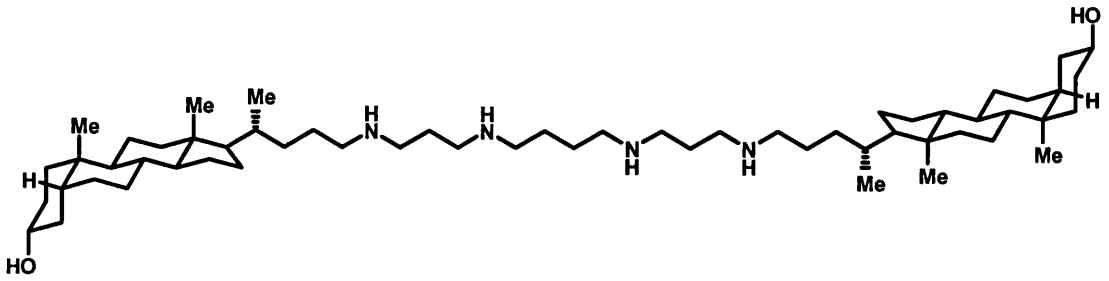
5.57 (2 H, m, 6'-CH, NH). ^{13}C NMR: 12.1 (18'-CH_3), 16.3 (19'-CH_3), 17.7 (28'-CH_3), 19.7 (27'-CH_3), 20.0 (26'-CH_3), 21.1 (11'-CH_2), 21.2 (21'-CH_3), 23.1 (15'-CH_2), 27.1 (2-CH_2), 28.3 (16'-CH_2), 28.6 (2'-CH_2), 33.1 (25'-CH), 37.1 (10'-C), 37.2 (4'-CH_2), 38.0 (1'-CH_2), 39.1 (12'-CH_2), 39.8 (1-CH_2), 40.4 (20'-CH), 42.8 (13'-C, 24'-CH overlapping), 45.2 ($(CH_3)_2N$), 46.1 (9'-CH), 54.5 (14'-CH), 55.7 (17'-CH), 57.6 (3-CH_2), 72.8 (3'-CH), 116.2 (7'-CH), 119.8 (6'-CH), 131.8 (23'-CH), 135.4 (22'-CH), 138.7 (8'-C), 141.1 (5'-C), 156.0 (N-CO-O). FAB-MS *m/z* 525 (MH^+), $C_{34}H_{56}N_2O_2$ requires 524; FAB-HRMS calculated 525.4420 (MH^+), found 525.4421.



N^1,N^{12}-Di-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-N^4,N^9-(di-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 35

EDC (76 mg, 0.54 mmol) and lithocholic acid (142 mg, 0.38 mmol) were dissolved in anhydrous CH_2Cl_2 (8 ml) and HOBt (10 mg, 74 μmol) was added with stirring at 25 °C under nitrogen. Di-Boc protected polyamine **34** (72 mg, 0.18 mmol) was added and the solution stirred for 16 h. CH_2Cl_2 (30 ml) was added and the solution was washed with water (50 ml) and saturated $NaHCO_3$ solution (60 ml). The organic layers were dried ($MgSO_4$), evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 30:1 v/v) to afford the title compound as a white foam (134 mg, 67 %). R_f 0.21 (CH_2Cl_2-MeOH 30:1 v/v). ^1H NMR ($CDCl_3$): 0.64 (6 H, s, 18'-CH_3 x 2), 0.91 (6 H, s, 19'-CH_3 x 2), 0.92 (6 H, d, J = 6, 21'-CH_3 x 2),

0.94-2.40 (84 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2 x 2, 2'-CH_2 x 2, 4'-CH_2 x 2, 5'-CH x 2, 6'-CH_2 x 2, 7'-CH_2 x 2, 8'-CH x 2, 9'-CH x 2, 11'-CH_2 x 2, 12'-CH_2 x 2, 14'-CH x 2, 15'-CH_2 x 2, 16'-CH_2 x 2, 17'-CH x 2, 20'-CH x 2, 22'-CH_2 x 2, 23'-CH_2 x 2, O-C(CH_3)$_3$ x 2), 3.10-3.30 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 10-CH_2, 9-CH_2, 12-CH_2), 3.58-3.66 (2 H, m, 3'-CH x 2), 6.83-6.93 (2 H, m, CH_2-N<u>H</u>-CO x 2). ^{13}C NMR: 12.1 (18'-CH_3 x 2), 18.4 (21'-CH_3 x 2), 20.9 (11'-CH_2 x 2), 23.4 (19'-CH_3 x 2), 24.2 (15'-CH_2 x 2), 25.5, 26.2 (6-CH_2, 7-CH_2), 26.5 (7'-CH_2 x 2), 27.2 (6'-CH_2 x 2), 27.6, 27.6 (2-CH_2, 11-CH_2), 28.2 (16'-CH_2 x 2), 28.4 (O-C(<u>C</u>H_3)$_3$ x 2), 30.5 (2'-CH_2 x 2), 31.9 (22'-CH_2 x 2), 33.7 (23'-CH_2 x 2), 34.6 (10'-C x 2), 35.4 (1'-CH_2 x 2), 35.5 (20'-CH x 2), 35.9 (8'-CH x 2), 36.4 (4'-CH_2 x 2), 37.4, 37.4 (1-CH_2, 12-CH_2), 40.2 (12'-CH_2 x 2), 40.4 (9'-CH x 2), 42.1 (5'-CH x 2), 42.7 (13'-C x 2), 44.8, 44.8, 46.5, 46.6, 46.8 (3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2), 56.0 (17'-CH x 2), 56.5 (14'-CH x 2), 71.6 (3'-CH x 2), 79.7 (O-<u>C</u>(CH_3)$_3$ x 2), 156.0 156.0 (O-<u>C</u>O-NH x 2), 173.6 (NH-<u>C</u>O x 2). FAB-MS *m/z* 1120 (MH^+), $C_{68}H_{118}N_4O_8$ requires 1118; FAB-HRMS calculated 1119.9028 (MH^+), found 1119.9067.



N^1,N^{12}-Di-(3'α-hydroxy-5'β-cholan-24'-yl)-1,12-diamino-4,9-diazadodecane 36

Amide **35** (105 mg, 0.09 mmol) in anhydrous CH_2Cl_2 (9 ml) and TFA (1 ml) was stirred at 25 °C for 2 h, then the solvent was evaporated *in vacuo* and the residue was recrystallised once from ethyl acetate to afford a crude white solid. To a refluxing solution of this solid in THF (20 ml)

was added Borane-DMS complex (10 M solution in DMS, 1 ml, 10 mmol) dropwise over 5 min to a stirred. The solution was stirred at reflux for 12 h (a white precipitate formed), then dilute aq. HCl (5 M, 10 ml) was added slowly (precipitate dissolved). The solution was stirred at reflux for a further 2 h then evaporated to dryness *in vacuo*. The residue was recrystallised from ethyl acetate to afford the title compound as a white solid (26 mg, 31 %). ^{1}H NMR (d_6-DMSO): 0.63 (6 H, s, 18'-CH_3 x 2), 0.87-0.91 (12 H, m, 19'-CH_3 x 2, 21'-CH_3 x 2), 0.93-2.35 (64 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2 x 2, 2'-CH_2 x 2, 4'-CH_2 x 2, 5'-CH x 2, 6'-CH_2 x 2, 7'-CH_2 x 2, 8'-CH x 2 9'-CH x 2, 11'-CH_2 x 2, 12'-CH_2 x 2, 14'-CH x 2, 15'-CH_2 x 2, 16'-CH_2 x 2, 17'-CH x 2, 20'-CH x 2, 22'-CH_2 x 2, 23'-CH_2 x 2), 2.82-3.36 (16 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 24-CH_2 x 2), 3.32-3.42 (2 H, m, 3'-CH x 2), 9.02-9.30 (2 x bs, ammonium NH). FAB-MS *m/z* 892 (MH^+), $C_{58}H_{106}N_4O_2$ requires 891; FAB-HRMS calculated 891.8382 (MH^+), found 891.8394.

3-Benzyloxycarbonylaminopropan-1-ol 40

To a stirred solution of 3-aminopropan-1-ol **38** (2.0 g, 27.4 mmol) in CH_2Cl_2 (200 ml) and TEA (3.8 ml, 27.4 mmol) at 25 °C, benzyl chloroformate (5.1 g, 30.1 mmol) was added dropwise over 5 min. The solution was stirred for 4 h, then evaporated to dryness *in vacuo* and the residue was purified over silica gel (EtOAc-hexane 6:4 v/v) to afford the title compound as a white solid (5.27 g, 92 %). R_f 0.41 (EtOAc-hexane 8:2 v/v). mp: 50-51 °C. IR 3326 cm^{-1} (OH), 1682 cm^{-1} (N-CO-O). ^{1}H NMR ($CDCl_3$): 1.68 (2 H, quin, J = 6, 2-CH_2), 3.10 (1 H, bs, CH_2-O<u>H</u>), 3.31 (2 H, q, J = 6, 3-CH_2), 3.64 (2 H, t, J = 6, 1-CH_2), 5.08 (2 H, s, O-CH_2-Ph), 5.27 (1 H, bs, CH_2-N<u>H</u>-CO-O), 7.27-7.37 (5 H, m, Ph). ^{13}C NMR: 32.9 (2-CH_2), 38.2 (3-CH_2), 59.9 (1-CH_2), 67.2 (O-CH_2-Ph), 128.3, 128.4, 128.7 (Ph), 136.6 (C_q Ph), 157.5 (N-<u>C</u>O-O). FAB-MS *m/z* 210 (MH^+), $C_{11}H_{15}NO_3$ requires 209; FAB-HRMS calculated 210.1130 (MH^+), found 210.1136.

6-Benzyloxycarbonylaminohexan-1-ol 41

To a stirred solution of 6-aminohexan-1-ol **39** (3.0 g, 26 mmol) in CH_2Cl_2 (250 ml) and TEA (3.6 g, 26 mmol) at 25 °C, benzyl chloroformate (4.85 g, 29 mmol) was added dropwise over 5 min. The solution was stirred for 18 h, then evaporated to dryness *in vacuo* and the residue was purified over silica gel (EtOAc-hexane 9:1 v/v) to afford the title compound as a white solid (6.31 g, 97 %). R_f 0.50 (EtOAc-hexane 8:2 v/v). mp: 78-80 °C. ^{1}H NMR ($CDCl_3$): 1.33-1.34 (4 H, m, 3-CH_2, 4-CH_2), 1.48-1.56 (4 H, m, 2-CH_2, 5-CH_2), 1.95 (1 H, s, CH_2-O<u>H</u>), 3.18 (2 H, q, J = 7, 6-CH_2), 3.60 (2 H, t, J = 7, 1-CH_2), 4.91 (1 H, bs, CH_2-N<u>H</u>-CO-O), 5.08 (2 H, s, O-CH_2-Ph), 7.30-7.35 (5 H, m, Ph). ^{13}C NMR: 25.2 (3-CH_2), 26.3 (4-CH_2), 29.8 (5-CH_2), 32.4 (2-CH_2), 40.8 (6-CH_2), 62.5 (1-CH_2), 66.5 (O-CH_2-Ph), 128.0, 128.4 (Ph), 136.5 (C Ph), 156.4 (N-<u>C</u>O-O). FAB-MS *m/z* 252, (MH^+), $C_{14}H_{21}NO_3$ requires 251; FAB-HRMS calculated found 252.1560 (MH^+), found 252.1604.

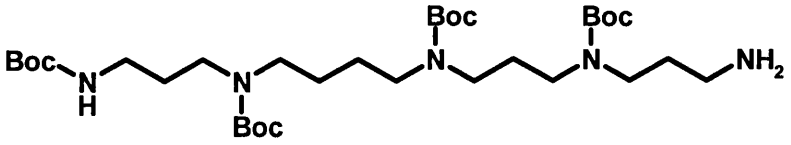
3-Benzyloxycarbonylaminopropanal 42

Oxalyl chloride (0.85 ml, 9.7 mmol) was dissolved in anhydrous CH_2Cl_2 (35 ml) and stirred at -78 °C under nitrogen. Anhydrous DMSO (1.25 ml, 17.6 mmol) was added dropwise over 5 min and the mixture stirred for a further 10 min. Alcohol **40** (1.84 g, 8.8 mmol) in anhydrous CH_2Cl_2 (40 ml) was added dropwise over 5 min. This gave a cloudy suspension which was allowed to warm to -20 °C until clear, then stirred at -78 °C for a further 10 min. TEA (6.1 ml, 44 mmol) was added and the solution warmed to 25 °C. Water (100 ml) was added and the organic layer was collected. The aqueous layer was extracted with CH_2Cl_2 (2 x 60 ml) and the combined organic extracts were dried ($MgSO_4$) and then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 3:1 v/v) to afford the title compound as a yellow oil (1.17 g, 64 %). R_f 0.51 (EtOAc-hexane 8:2 v/v). IR (thin film) 1713 cm^{-1} (CHO), 1684

cm^{-1} (N-CO-O). ^1H NMR ($CDCl_3$): 2.71 (2 H, t, J = 7, 2-CH_2), 3.40-3.51 (2 H, m, 3-CH_2), 5.08 (2 H, s, O-CH_2-Ph), 5.27 (1 H, bs, CH_2-NH-CO-O), 7.27-7.37 (5 H, m, Ph), 9.78 (1 H, s, CHO). ^{13}C NMR: 34.9 (2-CH_2), 44.2 (3-CH_2), 67.1 (O-CH_2-Ph), 128.3, 128.4, 128.7 (Ph), 136.6 (C Ph), 156.4 (N-CO-O), 201.3 (CHO). FAB-MS *m/z* 208 (MH^+), $C_{11}H_{13}NO_3$ requires M^+ = 207.

6-Benzyloxycarbonylaminohexanal 43

Oxalyl chloride (0.55 ml, 6.3 mmol) was dissolved in anhydrous CH_2Cl_2 (25 ml) and stirred at -78 °C under nitrogen. Anhydrous DMSO (0.8 ml, 11 mmol) was added dropwise over 4 min and the mixture stirred for a further 10 min. Alcohol **41** (1.43 g, 5.7 mmol) in anhydrous CH_2Cl_2 (30 ml) was added dropwise over 5 min. This gave a cloudy suspension which was allowed to warm to -20 °C until clear, then stirred at -78 °C for a further 10 min. TEA (4 ml, 28 mmol) was added and the solution warmed to 25 °C. Water (100 ml) was added and the organic layer was collected. The aqueous layer was extracted with CH_2Cl_2 (2 x 50 ml) and the combined organic extracts were dried ($MgSO_4$) and then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 3:7 v/v) to afford the title compound as a yellow oil (0.99g, 70 %). R_f 0.59 (EtOAc-hexane 8:2 v/v). IR (thin film) 1711 cm^{-1} (CHO), 1681 cm^{-1} (N-CO-O). ^1H NMR ($CDCl_3$): 1.31 (2 H, m, 4-CH_2), 1.50 (2 H, quin, J = 7, 5-CH_2), 1.61 (2 H, quin, J = 7, 3-CH_2), 2.40 (2 H, t, J = 7, 2-CH_2), 3.16 (2 H, q, J = 7, 6-CH_2), 5.07-5.16 (3 H, m, O-CH_2-Ph, CH_2-NH-CO-O), 7.28-7.34 (5 H, m, Ph), 9.72 (1 H, s, CHO). ^{13}C NMR: 21.6 (3-CH_2), 26.2 (4-CH_2), 29.7 (5-CH_2), 40.7 (2-CH_2), 43.6 (6-CH_2), 66.5 (O-CH_2-Ph), 128.0, 128.4 (Ph), 136.6 (C_q Ph), 156.5 (N-CO-O), 202.6 (CHO). FAB-MS *m/z* 250 (MH^+), $C_{14}H_{19}NO_3$ requires 249; FAB-HRMS calculated 250.1443 (MH^+), found 250.1443.



N^1,N^4,N^9,N^{13}-(Tetra-*tert*-butyloxycarbonyl)-1,16-diamino-4,9,13-triazahexadecane 46

Tri-Boc protected polyamine **5** (700 mg, 1.4 mmol) and aldehyde **42** (288 mg, 1.4 mmol) in anhydrous MeOH (25 ml) was stirred at 25 °C, under nitrogen, over 4 Å molecular sieves for 5 min. Glacial acetic acid (catalytic amount, 4 drops to pH 5) followed by sodium cyanoborohydride (151 mg, 2.4 mmol) were then added and the reaction mixture stirred for 24 h. The reaction mixture was filtered and then evaporated to dryness *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to give a colourless oil (312 mg, 32 %). R_f 0.32 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). This oil was dissolved in CH_2Cl_2 (15 ml) then TEA (1 ml, 7.7 mmol) and di-*tert*-butyl dicarbonate (144 mg, 0.66 mmol) were added and the solution stirred at 25 °C for 3 h. After this time, the solution was evaporated to dryness *in vacuo* and the residue redissolved in MeOH (15 ml) and Pearlman's catalyst (1 g, $Pd(OH)_2$, 20 % on carbon) was added. The reaction vessel was flushed with hydrogen (x 3) and stirred under an atmosphere of hydrogen for 18 h. The reaction mixture was then filtered and the filtrate concentrated *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford the title compound as a yellow oil (304 mg, 30%). R_f 0.26 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR ($CDCl_3$): 1.42-1.52 (40 H, m, 6-CH_2, 7-CH_2, O-C(CH_3)$_3$ x 4), 1.60-1.80 (4 H, m, 2-CH_2, 11-CH_2), 2.32-2.48 (2 H, bs, NH_2), 2.72 (2 H, t, J = 6, 16-CH_2), 3.05-3.35 (14 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2), 5.30-5.40 (1 H, bs, 1-CH_2-N<u>H</u>-CO-O). ^{13}C NMR: 25.6, 25.7, 25.7, 25.8, 25.9, 26.0 (6-CH_2, 7-CH_2, 11-CH_2), 28.5, 28.5 (2-CH_2, O-C(<u>C</u>H_3)$_3$ x 4, overlapping), 32.0 (15-CH_2), 37.5, 37.6 (1-CH_2), 38.6, 39.1 (16-CH_2), 43.9, 44.2, 44.6, 44.8 (3-CH_2, 10-CH_2, 12-CH_2, 14-

CH_2, overlapping), 46.8, 46.8 (5-CH_2, 8-CH_2 overlapping), 79.7, 79.8 (O-$\underline{C}(CH_3)_3$ x 4), 155.2, 158.1 (N-CO-O x 4, overlapping). FAB-MS *m/z* 660 (MH^+), $C_{33}H_{65}N_5O_8$ requires 659; FAB-HRMS calculated 660.4911 (MH^+), found 660.4908.

N^1,N^4,N^9,N^{13}-(Tetra-*tert*-butyloxycarbonyl)-1,19-diamino-4,9,13-triazanonadecane 47

Tri-Boc protected polyamine **5** (817 mg, 1.6 mmol) and aldehyde **43** (405 mg, 1.6 mmol) in anhydrous MeOH (25 ml) was stirred at 25 °C, under nitrogen, over 4 Å molecular sieves for 5 min. Glacial acetic acid (catalytic amount, 4 drops to pH 5) followed by sodium cyanoborohydride (153 mg, 2.4 mmol) were then added and the reaction mixture stirred for 24 h. The reaction mixture was filtered and then evaporated to dryness *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to give a colourless oil (494 mg, 41 %). R_f 0.35 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). This oil was dissolved in CH_2Cl_2 (15 ml) then TEA (1 ml, 7.7 mmol) and di-*tert*-butyl dicarbonate (218 mg, 1.0 mmol) were added and the solution stirred at 25 °C for 18 h. After this time, the solution was evaporated to dryness *in vacuo* and the residue (crude **45**) redissolved in MeOH and conc. aq. ammonia (10 ml) was added and stirred for 1 h. The solvent was evaporated *in vacuo* and the residue redissolved in MeOH (15 ml) and Pearlman's catalyst (1 g, $Pd(OH)_2$, 20 % on carbon) was added. The reaction vessel was flushed with hydrogen (x 3) and stirred under an atmosphere of hydrogen for 18 h. The reaction mixture was then filtered and the filtrate concentrated *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford the title compound as a yellow oil (384 mg, 34 % overall). R_f 0.28 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^1H NMR ($CDCl_3$): 1.22-1.34 (4 H, m, 16-CH_2, 17-CH_2), 1.41-1.52 (44 H, m, 6-CH_2, 7-CH_2, 15-CH_2, 18-CH_2, O-$C(CH_3)_3$ x 4), 1.56-1.80 (4 H, m, 2-CH_2, 11-CH_2), 2.35-2.49 (2 H, bs, NH_2), 2.65 (2 H, t, J = 6, 16-CH_2), 3.07-3.30 (14 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2), 5.33-5.43 (1 H, bs, NH-CO-O). ^{13}C

NMR: 25.5, 25.8 26.7, 26.9 (6-CH_2, 7-CH_2, 11-CH_2, 15-CH_2, 16-CH_2, 17-CH_2, overlapping), 27.9, 28.0, 28.3, 28.9 (2-CH_2, O-C($\underline{C}H_3$)$_3$ x 4, overlapping), 32.9 (18-CH_2), 37.6 (1-CH_2), 41.5, 41.7 (19-CH_2), 44.2, 44.5, 44.6 46.7, 46.8, 47.2 (3-CH_2 ,5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 14-CH_2), 79.8 (O-C-($\underline{C}H_3$)$_3$ x 4), 156.6, 156.8 (N-CO-O x 4, overlapping). FAB-MS *m/z* 702 (MH^+), $C_{36}H_{71}N_5O_8$ requires 701; FAB-HRMS calculated 702.5381 (MH^+), found 702.5390.

For characterisation, an analytical sample (100 mg) of **45** was purified over silica gel (EtOAc-hexane 6:4 v/v) to afford a yellow oil. R_f 0.84 (EtOAc v/v). ^1H NMR ($CDCl_3$): 1.23-1.34 (4 H, m, 3-CH_2, 4-CH_2), 1.39-1.54 (42 H, m, 2-CH_2, 5-CH_2, 13-CH_2, 14-CH_2, O-C(CH_3)$_3$ x 4), 1.79-1.82 (4 H, m, 9-CH_2, 18-CH_2), 3.00-3.27 (16 H, m, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2), 5.04-5.10 (2 H, m, O-C$\underline{H}_2$-Ph), 5.08-5.14 (1 H, s, 19-CH_2-N$\underline{H}$-CO-O), 5.30-5.38 (1 H, bs, 1-CH_2-N$\underline{H}$-CO-O), 7.22-7.40 (5 H, m, Ph). ^{13}C NMR ($CDCl_3$): 26.4, 27.2, 27.4, 27.6 (2-CH_2, 3-CH_2, 4-CH_2, 5-CH_2, 13-CH_2, 14-CH_2, O-C-($\underline{C}H_3$)$_3$ x 4, overlapping), 28.1, 28.2, 28.5, 29.9 (13-CH_2, 18-CH_2), 41.2, 41.3, 41.4, 41.5, 41.7 (1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2), 66.5 (O-$\underline{C}H_2$-Ph), 79.7 (O-C-($\underline{C}H_3$)$_3$ x 4), 128.1, 128.5 (Ph), 136.7 (C Ph), 155.5, 156.4 (N-CO-O x 5, overlapping). FAB-MS *m/z* 836 (MH^+), $C_{44}H_{77}N_5O_{10}$ requires 835; FAB-HRMS calculated 836.5749 (MH^+), found 836.5747.

N^1-(Cholesteryl-3'-oxycarbonyl)-N^4,N^8,N^{13},N^{16}-(tetra-*tert*-butyloxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 48

Cholesteryl chloroformate (323 mg, 0.72 mmol) in CH_2Cl_2 (5 ml) was added over 5 min to a stirred solution of tetra-Boc protected polyamine **46** (431 mg, 0.65 mmol) in CH_2Cl_2 (5 ml) with TEA (1 ml, 7.7 mmol) at 25 °C, under nitrogen. The solution was stirred for 18 h then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 4:6 to 5:5 v/v) to afford the title compound as a white solid (431 mg, 61 %). R_f 0.70 (EtOAc-hexane 7:3 v/v). ^1H NMR ($CDCl_3$): 0.66 (3 H, s, 18'-CH_3), 0.84, 0.85 (6 H, 2 x d, J = 7, overlapping 2

Hz, 26'-CH_3 and 27'-CH_3), 0.90 (3 H, d, *J* = 7, 21'-CH_3), 0.99 (3 H, s, 19'-CH_3), 1.02-2.03 (72 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 4), 2.22-2.37 (2 H, m, 24'-CH_2), 3.00-3.34 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2), 4.41-4.52 (1 H, m, 3'-CH), 5.35 (1 H, d, *J* = 4, 6'-CH), 5.47-5.60 (2 H, m, CH_2-N<u>H</u>-CO x 2). ^{13}C NMR: 12.0 (18'-CH_3), 18.8 (21'-CH_3), 19.4 (19'-CH_3), 21.1 (11'-CH_2), 22.6 (27'-CH_3), 22.9 (26'-CH_2), 23.9 (23'-CH_2), 24.4 (15'-CH_2), 25.4, 25.6, 25,9 (6-CH_2, 10-CH_2, 11-CH_2), 28.1, 28.2, 28.3, 28.5, 28.5 (2-CH_2, 15-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 4, overlapping), 32.0 (7'-CH_2, 8'-CH, overlapping), 35.7 (20'-CH), 36.2 (22'-CH_2); 36.6 (10'-C), 37.1 (1'-CH_2), 37.3 (1-CH_2, 16-CH_2), 38.6 (24'-CH_2), 39.6, 39.8 (4'-CH_2, 12'-CH_2), 42.4 (13'-C), 44.8, 46.6, 46.9 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2 overlapping), 50.1 (9'-CH), 56.2 (17'-CH), 56.7 (14'-CH_2), 74.1 (3'-CH), 79.2 (O-<u>C</u>(CH_3)$_3$ x 4), 122.3 (6'-CH), 139.7 (5'-C), 155.3, 156.0 (N-CO-O-<u>C</u>(CH_3)$_3$ x 4, NH-<u>C</u>O-O-CH, overlapping). FAB-MS *m/z* 1072 (MH^+), $C_{61}H_{109}N_5O_{10}$ requires 1071; FAB-HRMS calculated 1072.8253 (MH^+), found 1072.8272.

N^1-(Cholesteryl-3'-oxycarbonyl)-N^7,N^{11},N^{16},N^{19}-(tetra-*tert*-butyloxycarbonyl)-1,19-diamino-7,11,16-triazanonadecane 49

Cholesteryl chloroformate (85 mg, 0.19 mmol) in CH_2Cl_2 (5 ml) was added over 5 min to a stirred solution of tetra-Boc protected polyamine **47** (120 mg, 0.17 mmol) in CH_2Cl_2 (5 ml) with TEA (1 ml, 7.7 mmol) at 25 °C, under nitrogen. The solution was stirred for 18 h then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 4:6 v/v) to afford the title compound as a white solid (190 mg, 95 %). R_f 0.72 (EtOAc-hexane 7:3 v/v). ^{1}H NMR ($CDCl_3$): 0.68 (3 H, s, 18'-CH_3), 0.86, 0.87 (6 H, 2 x d, *J* = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.91 (3 H, d, *J* = 6, 21'-CH_3), 1.01 (3 H, s, 19'-CH_3), 1.04-2.04 (78 H, m, 2-CH_2, 3-CH_2, 4-CH_2, 5-CH_2, 9-CH_2, 13-CH_2, 14-CH_2, 18-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 7'-CH_2, 8'-CH,

9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 4), 2.22-2.39 (2 H, m, 24'-CH_2), 3.02-3.35 (16 H, m, 1-CH_2, 6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2, 19-CH_2), 4.43-4.55 (1 H, m, 3'-CH), 4.60-4.80 (2 H, m, CH_2-N<u>H</u>-CO x 2), 5.37 (1 H, d, *J* = 5, 6'-CH). ^{13}C NMR: 12.0 (18'-CH_3), 18.8 (21'-CH_3), 19.4 (19'-CH_3), 21.1 (11'-CH_2), 22.6 (27'-CH_3), 22.9 (26'-CH_2), 23.9 (23'-CH_2), 24.4 (15'-CH_2), 28.1, 28.3, 28.5, 28.6 (9-CH_2, 18-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C(<u>C</u>H_3)$_3$ x 3, overlapping), 32.0 (7'-CH_2, 8'-CH, overlapping), 35.8 (20'-CH), 36.2 (22'-CH_2); 36.6 (10'-C); 37.1 (1'-CH_2), 37.3 (19-CH_2), 38.6 (24'-CH_2), 39.6, 39.8 (4'-CH_2, 12'-CH_2), 40.8 (1-CH_2), 42.4 (13'-C), 44.8, 46.6, 46.9 (6-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 15-CH_2, 17-CH_2 overlapping), 46.9 (5-CH_2, 8-CH_2, overlapping), 50.1 (9'-CH), 56.2 (17'-CH), 56.7 (14'-CH_2), 74.1 (3'-CH), 79.2 (O-<u>C</u>-(CH_3)$_3$ x 3), 122.3 (6'-CH), 139.7 (5'-C), 155.3, 156.0 (N-<u>C</u>O-O-C(CH_3)$_3$ x 3, NH-<u>C</u>O-O-CH, overlapping). FAB-MS *m/z* 1115 (MH^+), $C_{64}H_{115}N_5O_{10}$ requires 1113; FAB-HRMS calculated 1114.8722 (MH^+), found 1114.8701.

N^1-(3'α-Hydroxy-5'β-cholan-24'-carbonyl)-N^4,N^8,N^{13},N^{16}-(tetra-*tert*-butyloxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 50

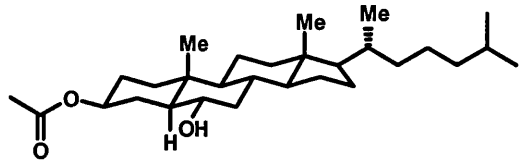
EDC (235 mg, 1.2 mmol) and lithocholic acid (231 mg, 0.61 mmol) were dissolved in CH_2Cl_2 (5 ml) and HOBt (17 mg, 0.13 mmol) was added with stirring at 25 °C under nitrogen. Tetra-Boc protected polyamine **46** (405 mg, 0.61 mmol) was added and the solution stirred for 16 h. CH_2Cl_2 (30 ml) was added and the solution was washed with water (50 ml) and saturated $NaHCO_3$ solution (60 ml). The organic layers were dried ($MgSO_4$), evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 30:1 to 20:1 v/v) to afford the title compound as a white foam (590 mg, 95%). R_f 0.30 (CH_2Cl_2-MeOH 20:1 v/v). ^{1}H NMR ($CDCl_3$): 0.64 (3 H, s, 18'-CH_3), 0.91 (3 H, s, 19'-CH_3), 0.92 (3 H, d, *J* = 6, 21'-CH_3), 0.95-2.30 (74 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-

CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, O-C(CH_3)$_3$ x 4), 3.04-3.33 (16 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2), 3.57-3.66 (1 H, m, 3'-CH), 5.31-5.40 (1 H, m, CH_2-N<u>H</u>-CO-O), 6.78-6.87 (1 H, m, CH_2-N<u>H</u>-CO). ^{13}C NMR: 12.1 (18'-CH_3), 18.4 (21'-CH_3), 20.9 (11'-CH_2), 23.4 (19'-CH_3), 24.3 (15'-CH_2), 25.5, 26.2 (10-CH_2, 11-CH_2), 26.5 (7'-CH_2), 27.2 (6'-CH_2), 27.6, 27.6 (2-CH_2, 6-CH_2, 15-CH_2), 28.3 (16'-CH_2), 28.5, 28.5 (O-C(<u>C</u>H_3)$_3$ x 4), 30.5 (2'-CH_2), 31.8 (22'-CH_2), 33.8 (23'-CH_2), 34.6 (10'-C), 35.4 (1'-CH_2), 35.5 (20'-CH), 35.9 (8'-CH), 36.4 (4'-CH_2) 37.4, 37.4 (1-CH_2, 16-CH_2), 40.2 (12'-CH_2), 40.4 (9'-CH), 42.1 (5'-CH), 42.7 (13'-C), 44.8, 44.8, 46.5, 46.6, 46.8 (3-CH_2, 5-CH_2, 7-CH_2, 12-CH_2, 14-CH_2), 56.0 (17'-CH), 56.4 (14'-CH), 71.7 (3'-CH), 79.4 (O-<u>C</u>(CH_3)$_3$ x 4), 156.0 156.1 (O-<u>C</u>O-NH), 173.5 (CO-NH). FAB-MS *m/z* 1018 (MH^+), $C_{49}H_{88}N_4O_8$ requires 1017; FAB-HRMS calculated 1018.7783 (MH^+), found 1018.7827.

Di-(3-benzyloxycarbonylaminopropan-1-yl) oxalate 51 (Attempted synthesis of 3-benzyloxycarbonylaminopropanal **42**)

Oxalyl chloride (0.92 ml, 10.6 mmol) was dissolved in anhydrous CH_2Cl_2 (30 ml) and stirred at -78 °C under nitrogen. DMSO (1.36 ml, 19.2 mmol) was added dropwise over 5 min and the mixture stirred for a further 10 min. Alcohol **40** (2.0 g, 9.6 mmol) in anhydrous CH_2Cl_2 (50 ml) was added dropwise over 5 min. This solution was stirred for a further 20 min. TEA (6.6 ml, 48 mmol) was added and the solution warmed to 25 °C. Water (100 ml) was added and the organic layer was collected. The aqueous layer was extracted with CH_2Cl_2 (3 x 80 ml) and the combined organic extracts were dried ($MgSO_4$) and then evaporated to dryness *in vacuo* and the residue purified over silica gel (EtOAc-hexane 7:3 v/v) to afford the title compound as a white solid (3.3 g, 73 %). R_f 0.52 (EtOAc-hexane 8:2 v/v). ^{1}H NMR ($CDCl_3$): 1.85-1.95 (4 H, m, 2-CH_2 x 2), 3.20-3.32 (4 H, m, 3-CH_2 x 2), 4.28 (4 H, t, J = 6, 1-CH_2 x 2), 5.06 (4 H, s, O-

C<u>H</u>$_2$-Ph x 2), 5.30-5.38 (2 H, m, CH$_2$-N<u>H</u>-CO-O x 2), 7.28-7.37 (10 H, m, Ph x 2). FAB-MS *m/z* 473 (MH^+), $C_{24}H_{28}N_2O_8$ requires M^+ = 472.



6'α-Hydroxy-5'α-cholesteryl-3'β-acetate 52

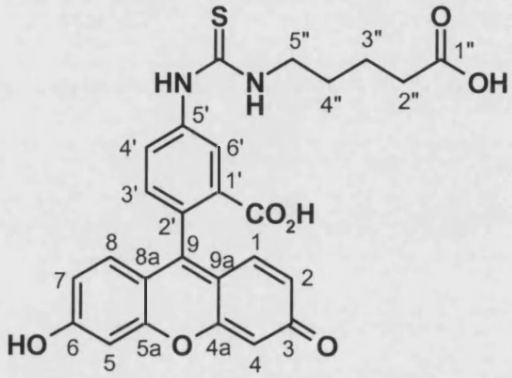
Borane-DMS complex (10 M solution in DMS, 0.6 ml, 5.6 mmol) was added dropwise over 5 min to a stirred solution of cholesteryl acetate (950 mg, 2.2 mmol) in anhydrous CH_2Cl_2 (10 ml), and EtOAc (1 ml) at 25 °C under nitrogen. The solution was stirred for 4 h then cooled to 0 °C in ice. Water (5 ml) was added and stirred for 5 min after which time H_2O_2 (30 % w/v aq. solution, 5 ml) and $NaHCO_3$ (saturated aq. solution, 5 ml) were added. The reaction mixture was stirred for 30 min then extracted with EtOAc (3 x 40 ml). The organic layers were combined, dried ($MgSO_4$), and evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 4:6 v/v) to afford the title compound as a white foam (672 g, 68 **%**). R_f 0.47 (EtOAc-hexane 4:6 v/v). ^{1}H NMR ($CDCl_3$): 0.64 (3 H, s, 18'-CH_3), 0.83 (3 H, s, 19'-CH_3), 0.86, 0.86 (6 H, 2 x d, *J* = 7, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.90 (3 H, d, *J* = 7, 21'-CH_3), 0.95-2.24 (33 H, m, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH_2, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 24'-CH_2 25'-CH, CH_3-CO), 3.39 (1 H, dt, *J* = 4 and 10, 6'-CH), 4.62-4.73 (1 H, m, 3'-CH). ^{13}C NMR: 12.1 (18'-CH_3), 13.5 (19'-CH_3), 18.6 (21'-CH_3), 21.2 (11'-CH_2), 22.7 (27'-CH_3), 22.9 (26'-CH_2), 23.9 (23'-CH_2), 24.3 (15'-CH_2), 27.3, 28.1, 28.3 (2'-CH_2, 16'-CH_2, CH_3-CO-O), 34.4 (8'-CH), 35.8 (20'-CH), 36.2, 36.3 (10'-C, 22-CH_2), 37.1 (1'-CH_2), 38.0 (24'-CH_2), 39.6, 39.8 (7'-CH_2, 12'-CH_2), 41.8 (4'-CH_2), 42.6 (13'-C), 51.7 (5'-CH), 53.7 (9'-CH), 56.1, 56.2 (14'-CH, 17'-CH), 69.5 (6'-CH) 73.6 (3'-CH), 170.4 (CH_3-C<u></u>O-O).

N^1-(6'α-Hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-N^4,N^9,N^{12}-(tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 54 and **N^1-(6'β-Hydroxy-5'β-cholesteryl-3'-oxycarbonyl)-N^4,N^9,N^{12}-(tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 55**

Borane-DMS complex (10 M solution in DMS, 0.3 ml, 2.6 mmol) was added dropwise over 5 min to a stirred solution of steroid **6** (950 mg, 1.0 mmol) in anhydrous CH_2Cl_2 (8 ml), at 25 °C under nitrogen. The solution was stirred for 4 h then cooled to 0 °C in ice. Water (5 ml) was added and stirred for 5 min after which time H_2O_2 (30 % aq. solution, 5 ml) and $NaHCO_3$ (saturated aq. solution, 5 ml) were added. The reaction mixture was stirred for 30 min then extracted with EtOAc (3 x 40 ml). The organic layers were combined, dried ($MgSO_4$), and evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 4:6 v/v) to afford compound **54** as a white foam (630 mg, 65 %). R_f 0.55 (EtOAc-hexane 7:3 v/v). ^1H NMR ($CDCl_3$): 0.61 (3 H, s, 18'-CH_3), 0.78 (3 H, s, 19'-CH_3), 0.82, 0.83 (6 H, 2 x d, J = 6, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.86 (3 H, d, J = 7, 21'-CH_3), 0.94-2.22 (64 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 24'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 3), 3.10-3.22 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 3.34 (1 H, dt, J = 4 and 10, 6'-CH), 4.46-4.58 (1 H, m, 3'-CH), 5.45-5.60 (2 H, m, CH_2-N<u>H</u>-CO x 2). ^{13}C NMR: 12.1 (18'-CH_3), 13.4 (19'-CH_3), 18.7 (21'-CH_3), 21.1 (11'-CH_2), 22.6 (27'-CH_3), 22.9 (26'-CH_2), 23.9 (23'-CH_2), 24.2 (15'-CH_2), 25.5, 26.1 (6-CH_2, 7-CH_2), 27.7, 28.0, 28.2, 28.5, 28.8 (2-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C(<u>C</u>H_3)$_3$ x 3, overlapping), 34.4 (8'-CH), 35.8 (20'-CH), 36.2 (10'-C), 36.3 (22'-CH_2), 37.1 (1'-CH_2), 37.3, 37.4 (1-CH_2, 12-CH_2), 38.9 (24'-CH_2), 39.5, 39.8 (7'-CH_2, 12'-CH_2), 42.8 (13'-C), 42.6 (4'-CH_2), 43.8, 44.0 (3-CH_2, 10-CH_2, overlapping), 46.7, 46.8 (5-CH_2, 8-CH_2, overlapping), 51.7 (5'-CH), 53.7 (9'-CH), 56.1 (14'-CH, 17'-CH_2), 69.4 (6'-CH), 73.8 (3'-CH), 79.5 (O-<u>C</u>(CH_3)$_3$ x 3), 155.9 (N-<u>C</u>O-O-C(CH_3)$_3$ x 3, NH-<u>C</u>O-O-CH,

overlapping). FAB-MS *m/z* 933 (MH^+), $C_{53}H_{94}N_4O_8$ requires 932; FAB-HRMS calculated 933.7256 (MH^+), found 933.7274.

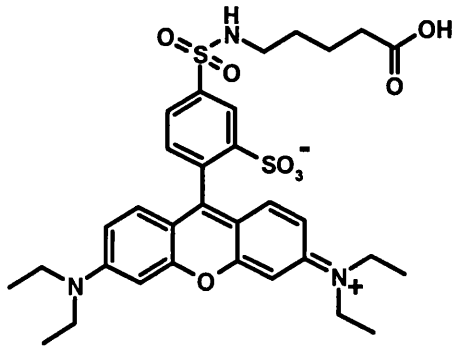
Further elution gave compound **55** as a white foam (30 mg, 3 %). R_f 0.14 (EtOAc-hexane 7:3 v/v). FAB-MS *m/z* 933 (MH^+), $C_{53}H_{94}N_4O_8$ requires 932; FAB-HRMS calculated 933.7256 (MH^+), found 933.7274.



5''-(Fluorescein-thioureido)-pentanoic acid 63

A solution of 5-aminopentanoic acid (66 mg, 0.51 mmol) in aq. NaOH (1M, 10 ml) was added dropwise over 5 min to a suspension of fluorescein isothiocyanate (200 mg, 0.51 mmol) in ethanol (4 ml). The solution was stirred for 5 h, then acidified to pH 3 with dilute aq. HCl to give a yellow precipitate which was collected by filtration and dried over P_2O_5 to afford the title compound as an orange-brown solid (252 mg, 95 %). t_R 12.9 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 495 nm, MeCN-0.1 % aq. TFA 8:2 v/v). ^1H NMR (CD_3OD): 1.14-1.16 (4 H, m, 3"-CH_2, 4"-CH_2), 2.37 (2 H, t, *J* = 7, 2"-CH_2), 3.60-3.67 (2 H, m, 5"-CH_2), 6.65 (2 H, dd, *J* = 9, 2, 2-CH, 7-CH), 6.78 (2 H, d, *J* = 2, 4-CH, 5-CH), 6.83 (2 H, d, *J* = 9, 1-CH, 8-CH), 7.18 (1 H, d, *J* = 8, 3'-CH), 7.80 (1 H, dd, *J* = 8, 2, 4'-CH), 8.22 (1 H, s, 6'-CH). ^{13}C NMR: 24.2 (3"-CH_2), 26.1 (4"-CH_2), 30.2 (2"-CH_2), 35.3 (5"-CH_2), 104.2, 113.3, 115.6, 122.0, 127.3, 130.1, 131.4, 131.6, 132.5, 143.0, 143.3, 155.9, 163.9 (1-CH, 2-CH, 3-C, 4-CH, 4a-C, 5-CH, 5a-C, 6-C, 7-

CH, 8-CH, 8a-C, 9-C, 9a-C, 1'-C, 2'-C, 3'-CH, 4'-CH, 5'-C, 6'-CH) 171.1 (1-C-COOH), 177.9 (1"-COOH), 183.2 (C=S). FAB-MS *m/z* 507 (MH^+), $C_{26}H_{22}N_2O_7S$ requires 506; FAB-HRMS calculated 507.1226 (MH^+), found 507.1218.



5''-(Lissamine rhodamine B sulfonamido)-pentanoic acid 64

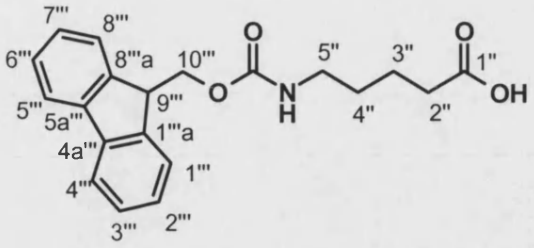
5-Aminopentanoic acid (61 mg, 0.52 mmol) in aq. NaOH (96.9 mM, 5.8 ml, 0.52 mmol) was added dropwise over 30 min to a stirred solution of lissamine rhodamine B sulfonyl chloride (300 mg, 0.52 mmol) in acetone (10 ml) at 25 °C. The solution was stirred for 16 h, then acidified to pH 3 with dilute aq. HCl. The solvent was evaporated *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 3:1 v/v) to afford the title compound as a purple solid (212 mg, 62 %). R_f 0.22 (CH_2Cl_2-MeOH 3:1 v/v). Absorption λ_{max} (MeCN): 554 nm (ε_{max} 57,416 $M^{-1}cm^{-1}$). ^1H NMR (D_2O): 1.08-1.21 (12 H, m, CH_3 x 4), 1.67-1.76 (4 H, m, 3"-CH_2, 4"-CH_2), 2.42-2.49 (2 H, m, 2"-CH_2), 3.00-3.06 (2 H, m, 5"-CH_2), 3.41-3.56 (8 H, m, CH_2-CH_3 x 4), 6.61 (2 H, s, 4-CH, 5-CH), 6.81 (2 H, d, *J* = 9, 2-CH, 7-CH), 6.94 (2 H, d, *J* = 9, 1-CH, 8-CH), 7.35 (1 H, d, *J* = 8, 3'-CH), 8.17 (1 H, d, *J* = 8, 4'-CH), 8.52 (1 H, s, 6'-CH). FAB-MS *m/z* 658 (MH^+), $C_{32}H_{39}N_3O_8S_2$ requires 657.

5''-Benzyloxycarbonylaminopentanoic acid 65

Benzyl chloroformate (2.00 g, 11.7 mmol) was added dropwise over 5 min to a stirred solution of 5-aminopentanoic acid (1.25 g, 10.7 mmol) in aq. NaOH (2 M, 100 ml) at 0 °C. The solution was stirred for 18 h, then acidified to pH 3 with dilute aq. HCl to give a white precipitate which was collected by filtration and dried over P_2O_5 to afford the title compound as a white solid (2.06 g, 77 %). mp: 98-99 °C. IR 1700 cm^{-1} (COOH), 1690 cm^{-1} (N-CO-O). ^1H NMR (d_6-DMSO): 1.41, 1.49 (4 H, 2 x quin, J = 7, overlapping 34 Hz, 3''-CH_2, 4''-CH_2), 2.21 (2 H, t, J = 7, 2''-CH_2), 2.99 (2 H, q, J = 6, 5''-CH_2), 3.60 (2 H, m, CH_2-N<u>H</u>-CO-O, COO<u>H</u> exchanged), 5.01 (s, 2 H, O-C<u>H</u>$_2$-Ph), 7.26-7.39 (5 H, m, Ph). ^{13}C NMR: 22.6 (3''-CH_2), 29.7 (4''-CH_2), 34.1 (2''-CH_2), 40.8 (5''-CH_2), 65.9 (O-<u>C</u>H_2-Ph), 128.3, 128.4, 129.0 (Ph), 137.9 (C Ph), 156.7 (N-<u>C</u>O-O), 175.0 (COOH). FAB-MS *m/z* 252 (MH^+), $C_{13}H_{17}NO_4$ requires 251; FAB-HRMS calculated 252.1236 (MH^+), found 252.1238.

5''-Benzyloxycarbonylaminopentanoyl chloride 65a

Oxalyl chloride (1.25 ml, 14.2 mmol) was added dropwise to a stirred solution of acid **65** (722 mg, 2.88 mmol) in toluene (10 ml) at 25 °C. The solution was stirred for 1 h then evaporated to dryness *in vacuo* to afford the title compound as a yellow oil (785mg, 86 % by ^{13}C NMR). IR (thin film) 1770 cm^{-1} (COCl), 1690 cm^{-1} (N-CO-O). ^{13}C NMR (d_6-DMSO): 19.9 (3''-CH_2), 22.2 (4''-CH_2), 34.5 (2''-CH_2), 46.2 (5''-CH_2), 67.6 (O-<u>C</u>H_2-Ph), 127.8, 128.4, 128.9 (Ph), 135.8 (C Ph), 153.8 (N-<u>C</u>O-O), 170.7 (COCl).



5''-(9'''-Fluorenylmethoxycarbonyl)-aminopentanoic acid 68

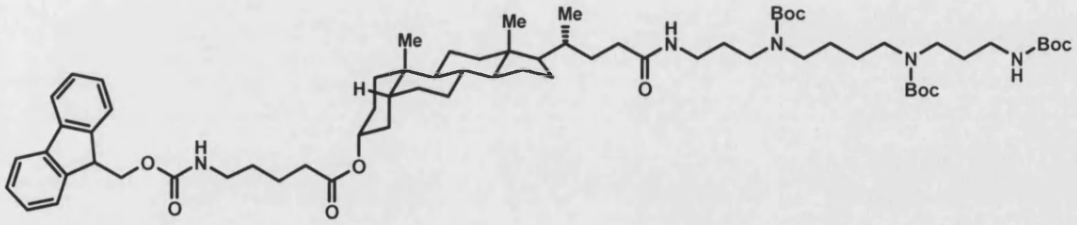
A solution of 5-aminopentanoic acid (930 mg, 8.0 mmol), *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (2.69 g, 8.0 mmol), $NaHCO_3$ (2.0 g) in water (20 ml) and dioxane (30 ml) was stirred for 16 h at 25 °C. The solution was washed with diethyl ether (3 x 50 ml). The aqueous layer was acidified to pH 3 with dilute aq. HCl, then extracted with EtOAc (3 x 60 ml). The combined organic extracts were evaporated to dryness *in vacuo*. The residue was recrystallised from EtOAc to afford the title compound as white crystals (2.22 g, 82 %). ^{1}H NMR (d_6-DMSO): 1.40 (2 H, quin, *J* = 7, 3''-CH_2), 1.48 (2 H, quin, *J* = 7, 4''-CH_2), 2.21 (2 H, t, *J* = 7, 2''-CH_2), 2.98 (2 H, q, *J* = 6, 5''-CH_2), 4.20 (1 H, t, *J* = 7, 9'''-CH), 4.29 (2 H, d, *J* = 7, 10'''-CH_2), 7.32 (2 H, t, *J* = 7, 2'''-CH and 7'''-CH), 7.40 (2 H, t, *J* = 7, 3'''-CH and 6'''-CH), 7.68 (2 H, d, *J* = 7, 1'''-CH and 8'''-CH), 7.88 (2 H, d, *J* = 7, 4'''-CH and 5'''-CH), 12.05 (1H, bs, COOH). ^{13}C NMR: 21.8 (3''-CH_2), 28.9 (4''-CH_2), 33.3 (2''-CH_2), 40.1 (5''-CH_2), 47.4 (9'''-CH), 65.1 (10'''-CH_2), 119.9 (2'''-CH and 7'''-CH), 125.0 (3'''-CH and 6'''-CH), 126.9 (1'''-CH and 8'''-CH), 127.4 (4'''-CH and 5'''-CH), 140.5 (4'''a-C and 5'''a-C), 143.7 (1'''a-C and 8'''a-C), 155.9 (O-CO-NH), 174.1 (COOH). FAB-MS *m/z* 340 (MH^+), $C_{20}H_{21}NO_4$ requires 339; FAB-HRMS calculated 340.1549 (MH^+), found 340.1547.

_N_-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1-aminopropane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester 69

5''-(9'''-Fluorenylmethoxycarbonyl)-aminopentanoic acid **68** (259 mg, 0.76 mmol) and DCC (174 mg, 0.84 mmol) were stirred in anhydrous CH_2Cl_2 (5 ml) and DMAP (20 mg, 0.16 mmol) was added. The solution was stirred for 1 min then secondary alcohol **67** (320 mg, 0.76 mmol) was added and the solution stirred for 6 h at 25 °C, and then evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 4:6 v/v) to afford the title compound as a white solid (482 mg, 85 %). R_f 0.62 (EtOAc-hexane 8:2 v/v). ^{1}H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.89-0.94 (9 H, m, 3-CH_3, 21'-CH_3, 19'-CH_3 overlapping), 0.97-2.34 (36 H, 2-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 2''-CH_2, 3''-CH_2, 4''-CH_2), 3.17-3.25 (4 H, m, 1-CH_2, 5''-CH_2), 4.21 (1 H, t, J = 7, C<u>H</u>-CH_2), 4.39 (2 H, d, J = 7, CH-C<u>H</u>$_2$), 4.69-4.80 (1 H, m, 3'-CH), 4.88-4.94 (1 H, m, CH_2-N<u>H</u>-CO-O), 5.46-5.52 (1 H, m, CH_2-N<u>H</u>-CO-CH_2), 7.31 (2 H, t, J = 7, 2'''-CH, 7'''-CH), 7.40 (2 H, t, J = 7, 3'''-CH, 6'''-CH), 7.59 (2 H, d, J = 7, 1'''-CH, 8'''-CH), 7.76 (2 H, d, J = 7, 4'''-CH, 5'''-CH). ^{13}C NMR: 11.5 (3-CH_3), 12.1 (18'-CH_3), 18.5 (21'-CH_3), 20.9 (11'-CH_2), 22.2 (3''-CH_2), 23.2 (2-CH_2), 23.4 (19'-CH_3), 24.2 (15'-CH_2), 26.4, 26.8, (7'-CH_2, 4''-CH_2), 27.1 (6'-CH_2), 28.3 (16'-CH_2), 29.4 (2'-CH_2), 31.9 (22'-CH_2), 32.4 (4'-CH_2), 34.0 (23'-CH_2), 34.3 (2''-CH_2), 34.6 (10'-C), 35.1 (1'-CH_2), 35.5 (20'-CH), 35.8 (8'-CH), 40.2 (12'-CH_2), 40.5 (9'-CH), 40.7 (5''-CH_2), 41.2 (1-CH_2), 42.0 (5'-CH), 42.8 (13'-C), 49.1 (O-CH_2-<u>C</u>H), 56.1 (17'-CH), 56.5 (14'-CH), 66.5 (O-<u>C</u>H_2-CH), 74.4 (3'-CH), 119.8 (2'''-CH, 7'''-CH), 124.9 (3'''-CH, 6'''-CH), 126.9 (1'''-CH, 8'''-CH), 127.5 (4'''-CH, 5'''-CH), 141.1 (4'''a-C, 5'''a-C), 143.8 (1'''a-C, 8'''a-C), 156.2 (O-CO-NH), 172.7 (O-<u>C</u>O-CH_2), 173.2 (CH_2-<u>C</u>O-NH). FAB-MS *m/z* 739 (MH^+), $C_{47}H_{66}N_2O_5$ requires 738; FAB-HRMS calculated 739.5050 (MH^+), found 739.5066.

4-Nitrophenyl 5''-(9'-fluorenylmethoxycarbonyl)-aminopentanoate 71

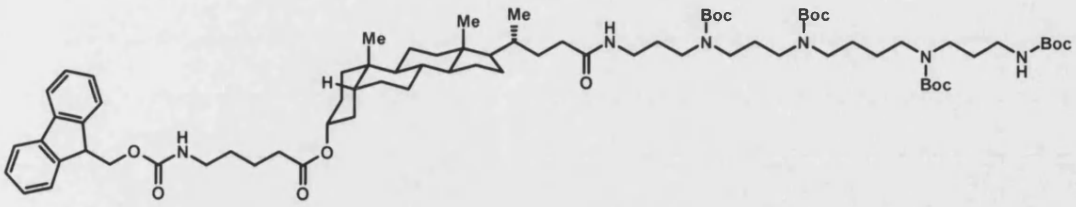
4-Nitrophenol (395 mg, 2.84 mmol) in EtOAc (5 ml) was added to a solution of DCC (586 mg, 2.84 mmol) and acid **68** (964 mg, 2.84 mmol) in EtOAc (30 ml) at 25 °C. The solution was stirred for 16 h then filtered. Then the filtrate was evaporated to dryness *in vacuo* and the residue was recrystallised once from EtOAc to afford the title compound as yellow crystals (967 mg, 74 %). ^{1}H NMR ($CDCl_3$): 1.65, 1.78 (4 H, 2 x quin, *J* = 7, 3''-CH_2, 4''-CH_2), 2.63 (2 H, t, *J* = 7, 2''-CH_2), 3.25 (2 H, q, *J* = 6, 5''-CH_2), 4.20 (1 H, t, *J* = 7, CH_2C<u>H</u>), 4.41 (2 H, d, *J* = 7, C<u>H</u>$_2$-CH), 7.25 (2 H, d, J = 9, 2-CH, 6-CH), 7.30 (2 H, t, *J* = 7, 2'''-CH, 7'''-CH), 7.39 (2 H, t, *J* = 7, 3'''-CH, 6'''-CH), 7.58 (2 H, d, *J* = 7, 1'''-CH, 8'''-CH), 7.75 (2 H, d, *J* = 7, 4'''-CH, 5'''-CH), 8.24 (2 H, d, *J* = 9, 3-CH, 5-CH). ^{13}C NMR: 21.8 (3''-CH_2), 29.4 (4''-CH_2), 33.8 (2''-CH_2), 40.5 (5''-CH_2), 47.3 (O-CH_2-<u>C</u>H), 66.6 (O-<u>C</u>H$_2$-CH), 119.8 (2'''-CH, 7'''-CH), 122.2 (2-CH, 6-CH), 124.8 (3-CH, 5-CH), 124.8 (3'''-CH, 6'''-CH), 126.9 (1'''-CH, 8'''-CH), 127.5 (4'''-CH, 5'''-CH), 141.1 (4'''a-C, 5'''a-C), 143.7 (1'''a-C, 8'''a-C), 145.1 (4''-C), 155.1, 156.3 (1''-C, O-<u>C</u>O-NH), 170.6 (O-<u>C</u>O-CH_2).



***N*^{4},*N*^{9},*N*^{12}-(Tri-*tert*-butyloxycarbonyl)-*N*^{1}-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,12-diamino-4,9-diazadodecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester 72**

5-(9-Fluorenylmethoxycarbonyl)-aminopentanoic acid **68** (70 mg, 0.21 mmol) and DCC (43 mg, 0.21 mmol) were stirred in anhydrous CH_2Cl_2 (5 ml) and DMAP (5 mg, 40 μmol) was added.

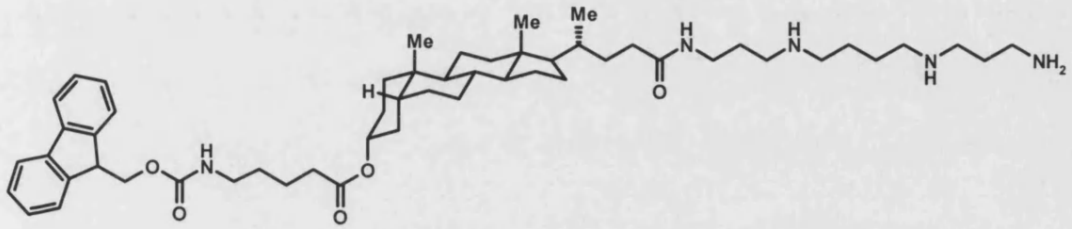
The solution was stirred for 1 min then secondary alcohol **16** (178 mg, 0.21 mmol) was added and the solution stirred for 18 h at 25 °C, and then evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 4:6 v/v) to afford the title compound as a white solid (195 mg, 80 %). R_f 0.45 (EtOAc-hexane 7:3 v/v). ^{1}H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.91 (3 H, d, J = 6, 21'-CH_3), 0.92 (3 H, s, 19'-CH_3), 0.98-2.34 (69 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 2''-CH_2, 3''-CH_2, 4''-CH_2, O-$C(CH_3)_3$ x 3), 3.00-3.32 (14 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 5''-CH_2), 4.21 (1 H, t, J = 7, C<u>H</u>-CH_2), 4.39 (2 H, d, J = 7, CH-C<u>H</u>$_2$), 4.69-4.78 (1 H, m, 3'-CH), 5.25-5.36 (1 H, m, CH_2-N<u>H</u>-CO-O), 6.73-6.82 (1 H, m, CH_2-N<u>H</u>-CO-CH_2), 7.31 (2 H, t, J = 7, 2'-CH, 7'-CH), 7.40 (2 H, t, J = 7, 3'-CH, 6'-CH), 7.59 (2 H, d, J = 7, 1'-CH, 8'-CH), 7.76 (2 H, d, J = 7, 4'-CH, 5'-CH). ^{13}C NMR: 12.1 (18'-CH_3), 18.5 (21'-CH_3), 20.9 (11'-CH_2), 22.2 (3''-CH_2), 23.4 (19'-CH_3), 24.2 (15'-CH_2), 25.7, 26.3 (6-CH_2, 7-CH_2), 26.8, 26.8, (7'-CH_2, 4''-CH_2, overlapping), 27.1 (6'-CH_2), 27.6, 27.6 (2-CH_2, 11-CH_2), 28.3, 28.5, 28.5 (16-CH_2, O-C(<u>C</u>$H_3)_3$ x 3,), 29.4 (2'-CH_2), 31.9 (22'-CH_2), 32.4 (4'-CH_2), 34.0 (23'-CH_2), 34.2 (2''-CH_2), 34.6 (10'-C), 35.1 (1'-CH_2), 35.5 (20'-CH), 35.8 (8'-CH), 37.4, 37.8 (1-CH_2, 12-CH_2), 40.2 (12'-CH_2), 40.4 (9'-CH), 40.5 (5''-CH_2), 41.9 (5'-CH), 42.8 (13'-C), 44.3, 46.7 (3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, overlapping), 49.3 (O-CH_2-<u>C</u>H), 56.1 (17'-CH), 56.4 (14'-CH), 66.5 (O-<u>C</u>H_2-CH), 74.3 (3'-CH), 79.7 (O-<u>C</u>$(CH_3)_3$ x 3), 119.8 (2'-CH, 7'-CH), 124.9 (3'-CH, 6'-CH), 126.8 (1'-CH, 8'-CH), 127.5 (4'-CH, 5'-CH), 141.1 (4a'-C, 5a'-C), 143.8 (1a'-C, 8a'-C), 156.2 (N-<u>C</u>O-O-$C(CH_3)_3$ x 3, N-<u>C</u>O-O-CH_2 overlapping), 173.0 (O-<u>C</u>O-CH_2), 173.7 (CH_2-<u>C</u>O-NH). FAB-MS *m/z* 1182 (MH^+), $C_{69}H_{107}N_5O_{11}$ requires 1181; FAB-HRMS calculated 1182.8045 (MH^+), found 1182.8025.



N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester 73

5-(9-Fluorenylmethoxycarbonyl)-aminopentanoic acid **68** (22 mg, 65 μmol) and DCC (19 mg, 93 μmol) were stirred in anhydrous CH_2Cl_2 (8 ml) and DMAP (2 mg, 16 μmol) was added at 25 °C. The solution was stirred for 1 min then secondary alcohol **50** (66 mg, 62 μmol) was added and the solution stirred for 28 h then evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 2:8 to 9:1 v/v) to afford the title compound as a white solid (38 mg, 44 %). R_f 0.53 (EtOAc-hexane 7:3 v/v). ^{1}H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.89-0.94 (6 H, m, 21'-CH_3 and 19'-CH_3), 0.96-2.45 (80 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH_2, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 2''-CH_2, 3''-CH_2, 4''-CH_2, O-C(CH_3)$_3$ x 4,), 3.00-3.34 (18 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2, 5''-CH_2), 4.21 (1 H, t, *J* = 7, C<u>H</u>-CH_2), 4.39 (2 H, d, *J* = 6, CH-C<u>H</u>$_2$), 4.69-4.78 (1 H, m, 3'-CH), 5.30-5.40 (1 H, m, CH_2-N<u>H</u>-CO-O), 6.74-6.88 (1 H, m, CH_2-N<u>H</u>-CO-CH_2), 7.31 (2 H, t, *J* = 7, 2'-CH, 7'-CH), 7.40 (2 H, t, *J* = 7, 3'-CH, 6'-CH), 7.59 (2 H, d, *J* = 7, 1'-CH, 8'-CH), 7.76 (2 H, d, *J* = 7, 4'-CH, 5'-CH). ^{13}C NMR: 12.2 (18'-CH_3), 18.5 (21'-CH_3), 20.9 (11'-CH_2), 22.2 (3''-CH_2), 23.4 (19'-CH_3), 24.3 (15'-CH_2), 25.6, 26.2 (10-CH_2, 11-CH_2), 26.4, 26.8, (7'-CH_2, 4''-CH_2), 27.1 (6'-CH_2), 27.6, 27.6 (2-CH_2, 6-CH_2, 15-CH_2), 28.3 (16'-CH_2), 28.5, 28.5 (O-C(<u>C</u>H_3)$_3$ x 4), 29.4 (2'-CH_2), 31.9 (22'-CH_2), 32.3 (4-CH_2), 33.9 (23'-CH_2), 34.2 (2''-CH_2), 34.6 (10'-C), 35.1 (1'-CH_2), 35.6 (20'-CH), 35.8 (8'-

CH), 37.4, 37.4 (1-CH_2, 16-CH_2), 40.1 (12'-CH_2), 40.4 (9'-CH), 40.6 (5''-CH_2), 41.9 (5'-CH), 42.7 (13'-C), 44.8, 44.8 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2), 47.3 (O-CH_2-CH), 56.1 (17'-CH), 56.4 (14'-CH), 66.5 (O-CH_2-CH), 74.4 (3'-CH), 79.4, 79.8 (O-$C(CH_3)_3$ x 4), 119.8 (2'-CH, 7'-CH), 124.9 (3'-CH, 6'-CH), 126.8 (1'-CH, 8'-CH), 127.5 (4'-CH, 5'-CH), 141.1 (4a'-C, 5a'-C), 143.8 (1a'-C, 8a'-C), 156.2 (N-CO-O-$C(CH_3)_3$ x 4, N-CO-O-CH_2 overlapping), 172.7 (O-CO-CH_2), 173.6 (CH_2-CO-NH). FAB-MS *m/z* 1339 (MH^+), $C_{77}H_{122}N_6O_{13}$ requires 1338.

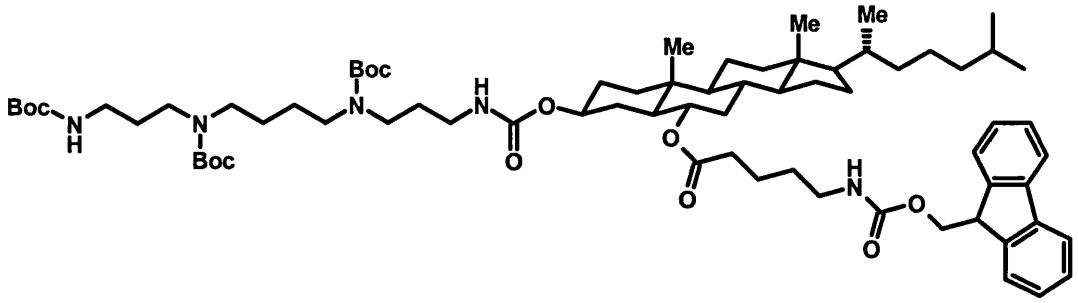


N^1-(3'α-5'β-Cholan-24'-carbonyl)-1,12-diamino-4,9-diazadodecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester tristrifluoroacetate salt 74

Tri-Boc protected polyamine **72** (25 mg) in anhydrous CH_2Cl_2 (18 ml) and TFA (2 ml) was stirred at 25 °C for 3 h, then the solvent was evaporated *in vacuo* and the residue purified by semi-preparative RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 265 nm, MeCN-0.1 % aq. TFA 8:2 v/v) to afford the title compound as a white solid (10 mg, 55 %). t_R 8.2 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 265 nm, MeCN-0.1 % aq. TFA 8:2 v/v). Absorption λ_{max} (H_2O): 262 nm. Fluorescence λ_{max} (Ex): 265 nm, λ_{max} (Em): 320 nm. FAB-MS *m/z* 882 (MH^+), $C_{54}H_{83}N_5O_5$ requires 881; FAB-HRMS calculated 882.6472 (MH^+), found 882.6444.

N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 75

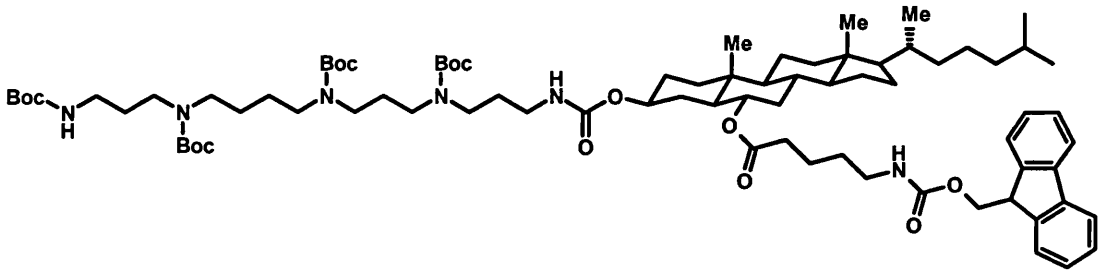
Borane-DMS complex (10 M solution in DMS, 0.1 ml, 0.70 mmol) was added dropwise over 5 min to a stirred solution of steroid **48** (300 mg, 0.28 mmol) in anhydrous CH_2Cl_2 (8 ml), at 25 °C under nitrogen. The solution was stirred for 4 h then cooled to 0 °C in ice. Water (5 ml) was added and stirred for 5 min after which time H_2O_2 (30 % aq. solution, 5 ml) and $NaHCO_3$ (saturated aq. solution, 5 ml) were added. The reaction mixture was stirred for 30 min then extracted with EtOAc (3 x 40 ml). The organic layers were combined, dried ($MgSO_4$), and evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 4:6 v/v) to afford the title compound as a white foam (195 mg, 64 %). R_f 0.61 (EtOAc-hexane 7: v/v). ^{1}H NMR ($CDCl_3$): 0.65 (3 H, s, 18'-CH_3), 0.82 (3 H, s, 19'-CH_3), 0.86, 0.87 (6 H, 2 x d, J = 6, overlapping 2 Hz, 26'-CH_3 and 27'-CH_3), 0.90 (3 H, d, J = 7, 21'-CH_3), 1.04-2.26 (75 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 24'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 4), 3.02-3.30 (16 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2), 3.38 (1 H, dt, J = 4 and 10, 6'-CH), 4.49-4.59 (1 H, m, 3'-CH), 5.34-5.68 (2 H, m, CH_2-N<u>H</u>-CO x 2). ^{13}C NMR: 12.4 (18'-CH_3), 13.8 (19'-CH_3), 19.1 (21'-CH_3), 21.5 (11'-CH_2), 23.0 (27'-CH_3), 23.2 (26'-CH_3), 24.2 (23'-CH_2), 24.6 (15'-CH_2), 25.8, 26.3 (6-CH_2, 10-CH_2, 11-CH_2), 28.0, 28.4, 28.6, 28.8, 28.9 (2-CH_2, 15-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C(<u>C</u>H_3)$_3$ x 4, overlapping), 34.7 (8'-CH), 36.1 (20'-CH), 36.5 (22'-CH_2), 36.6 (10'-C), 38.1 (1-CH_2, 16-CH_2), 37.4 (1'-CH_2), 38.0 (24'-CH_2), 39.8 (12'-CH_2), 40.1 (7'-CH_2), 42.0 (4'-CH_2), 42.9 (13'-C), 43.9, 44.0, 46.8, 47.0 (3-CH_2, 10-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2 overlapping), 52.0 (5'-CH), 54.0 (9'-CH), 56.5 (14'-CH, 17'-CH), 69.6 (6'-CH), 74.1 (3'-CH), 79.7, 79.9 (O-<u>C</u>-(CH_3)$_3$ x 4), 155.6, 156.3 (N-<u>C</u>O-O-C(CH_3)$_3$ x 4, NH-<u>C</u>O-O-CH, overlapping). FAB-MS *m/z* 1091 (MH^+), $C_{61}H_{111}N_5O_{11}$ requires 1089; FAB-HRMS calculated 1090.8358 (MH^+), found 1090.8371.



N^4,N^9,N^{12}-(Tri-*tert*-butyloxycarbonyl)-N^1-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,12-diamino-4,9-diazadodecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic ester 76

5-(9-Fluorenylmethoxycarbonyl)-aminopentanoic acid **68** (101 mg, 0.30 mmol) and DCC (62 mg, 0.30 mmol) were stirred in anhydrous CH_2Cl_2 (5 ml) and DMAP (7 mg, 0.06 mmol) was added at 25 °C. The solution was stirred for 1 min then secondary alcohol **54** (281 mg, 0.30 mmol) was added and the solution stirred for 18 h then evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 3:7 to 6:4 v/v) to afford the title compound as a white solid (312 mg, 83 %). R_f 0.66 (EtOAc-hexane 7:3 v/v). ^{1}H NMR ($CDCl_3$): 0.63 (3 H, s, 18'-CH_3), 0.86, 0.86 (6 H, 2 x d, overlapping 2 Hz, J = 6, 26'-CH_3 and 27'-CH_3), 0.83-0.92 (6 H, m, 19'-CH_3, 21'-CH_3), 0.93-2.45 (71 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 24'-CH_2, 25'-CH, 2''-CH_2, 3''-CH_2, 4''-CH_2, O-C(CH_3)$_3$ x 3), 3.10-3.31 (14 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2, 5''-CH_2), 4.22 (1 H, t, J = 6, 9'''-CH), 4.38 (2 H, d, J = 6, 10'''-CH_2), 4.42-4.55 (1 H, m, 3'-CH), 4.63-4.75 (1 H, m, 6'-CH), 5.29-5.68 (2 H, m, CH_2-N<u>H</u>-CO x 2), 7.31 (2 H, t, J = 7, 2'''-CH, 7'''-CH), 7.40 (2 H, t, J = 7, 3'''-CH, 6'''-CH), 7.61 (2 H, d, J = 7, 1'''-CH, 8'''-CH), 7.76 (2 H, d, J = 7, 4'''-CH, 5'''-CH). ^{13}C NMR: 12.1 (18'-CH_3), 13.3 (19'-CH_3), 18.7 (21'-CH_3), 21.3 (11'-CH_2), 22.4 (3''-CH_2), 22.7 (27'-CH_3), 22.9 (26'-CH_3), 24.0 (23'-CH_2), 24.2 (15'-CH_2), 25.0, 25.7, (6-CH_2, 10-CH_2, 11-CH_2, 4''-CH_2), 28.1, 28.2,

28.5, 28.9, 29.6 (2-CH_2, 11-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C($\underline{C}H_3$)$_3$ x 3, overlapping), 34.0, 34.1 (2''-CH_2, 8'-CH), 35.8 (20'-CH), 36.2 (22'-CH_2), 36.6 (10'-C), 37.4 (1'-CH_2), 37.7, 37.9 (1-CH_2, 12-CH_2, 24'-CH_2, overlapping), 39.5, 39.7 (7'-CH_2, 12'-CH_2), 40.7 (4'-CH_2, 5''-CH_2, overlapping), 42.6 (13'-C), 44.2, 46.8, 47.4 (3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, overlapping), 48.5 (9'''-CH), 49.1 (5'-CH_2), 53.5 (9'-CH), 56.1, 56.2 (14'-CH, 17'-CH), 66.6 (10'''-CH_2), 72.3 (6'-CH), 73.4 (3'-CH), 79.6 (O-$\underline{C}$(CH_3)$_3$ x 3), 119.8 (2'''-CH, 7'''-CH), 125.0 (3'''-CH, 6'''-CH), 126.9 (1'''-CH, 8'''-CH), 127.5 (4'''-CH, 5'''-CH), 141.1 (4'''a-C, 5'''a-C), 143.8 (1'''a-C, 8'''a-C), 156.3, 156.6 (N-$\underline{C}$O-O-C(CH_3)$_3$ x 3, N-$\underline{C}$O-O-CH_2 overlapping) 172.9 (CH-O-$\underline{C}$O-CH_2). FAB-MS *m/z* 1254 (MH^+), $C_{73}H_{115}N_5O_{12}$ requires 1254; FAB-HRMS calculated 1254.8621 (MH^+), found 1254.8664.

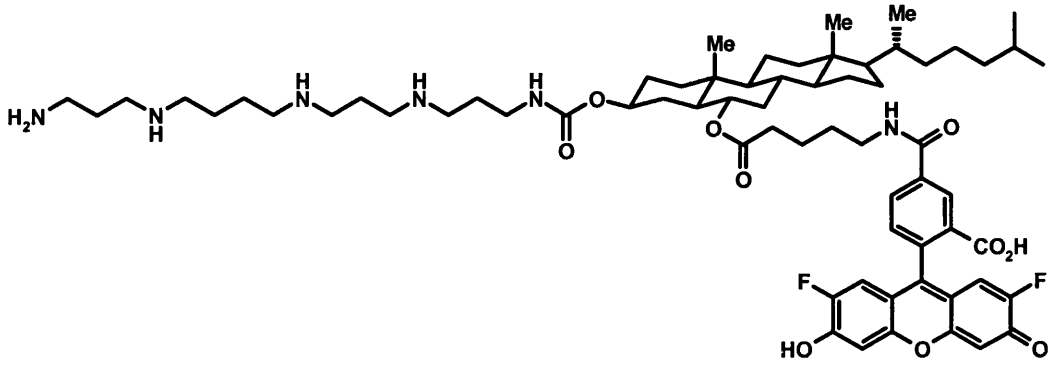


N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(9'''-fluorenylmethoxycarbonyl)-aminopentanoic acid ester 77

5-(9-Fluorenylmethoxycarbonyl)-aminopentanoic acid **68** (51 mg, 0.15 mmol) and DCC (19 mg, 0.15 mmol) were stirred in anhydrous CH_2Cl_2 (5 ml) and DMAP (3 mg, 0.02 mmol) was added at 25 °C. The solution was stirred for 1 min then secondary alcohol **75** (109 mg, 0.10 mmol) was added and the solution stirred for 26 h then evaporated to dryness *in vacuo*. The residue was purified over silica gel (EtOAc-hexane 2:8 to 4:6 v/v) to afford the title compound as a white solid (104 mg, 74 %). R_f 0.70 (EtOAc-hexane 7:3 v/v). ^{1}H NMR ($CDCl_3$): 0.63 (3 H, s,

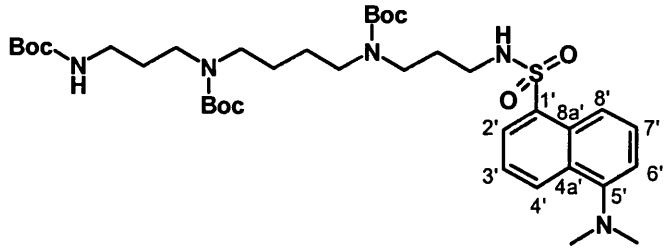
18'-CH_3), 0.86, 0.86 (6 H, 2 x d, overlapping 2 Hz, *J* = 6, 26'-CH_3 and 27'-CH_3), 0.88-0.92 (6 H, m, 21'-CH_3 and 19'-CH_3), 0.94-2.45 (82 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, 24'-CH_2, 25'-CH, 2''-CH_2, 3''-CH_2, 4''-CH_2, O-C(CH_3)$_3$ x 4), 3.00-3.35 (18 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2, 5''-CH_2), 4.22 (1 H, t, *J* = 6, 9'''-CH), 4.38 (2 H, d, *J* = 6, 10'''-CH_2), 4.44-4.55 (1 H, m, 3'-CH), 4.63-4.73 (1 H, m, 6'-CH), 5.30-5.66 (2 H, m, CH_2-NH-CO x 2), 7.31 (2 H, t, *J* = 7, 2'''-CH, 7'''-CH), 7.40 (2 H, t, *J* = 7, 3'''-CH, 6'''-CH), 7.59 (2 H, d, *J* = 7, 1'''-CH, 8'''-CH), 7.76 (2 H, d, *J* = 7, 4'''-CH, 5'''-CH).

^{13}C NMR: 12.4 (18'-CH_3), 13.6 (19'-CH_3), 19.1 (21'-CH_3), 21.5 (11'-CH_2), 22.2 (3''-CH_2), 23.0 (27'-CH_3), 23.2 (26'-CH_3), 24.3 (23'-CH_2), 24.5 (15'-CH_2), 25.9, 26.0, 26.6 (6-CH_2, 10-CH_2, 11-CH_2, 4''-CH_2,), 27.9, 28.4, 28.8, 28.9, 29.2 (2-CH_2, 15-CH_2, 2'-CH_2, 16'-CH_2, 25'-CH, O-C(CH_3)$_3$ x 4, overlapping), 34.4 (2''-CH_2) 34.7 (8'-CH), 36.1 (20'-CH), 36.5 (22'-CH_2), 36.9 (10'-C), 37.3 (1'-CH_2), 37.8, 37.9 (1-CH_2, 16-CH_2), 38.0 (24'-CH_2), 39.9 (12'-CH_2), 40.0 (7'-CH_2), 40.5 (5''-CH_2), 41.0 (4'-CH_2), 42.9 (13'-C), 44.2, 44.5, 45.1, 46.8, 47.3 (3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2 overlapping), 48.8 (9'''CH), 49.2 (5'-CH_2), 53.8 (9'-CH), 56.4, 56.5 (14'-CH, 17'-CH), 66.2 (10'''-CH_2), 72.7 (6'-CH), 74.0 (3'-CH), 79.7, 79.9, 80.0 (O-C-(CH_3)$_3$ x 4), 120.1 (2'''-CH, 7'''-CH), 125.2 (3'''-CH, 6'''-CH), 127.2 (1'''-CH, 8'''-CH), 127.8 (4'''-CH, 5'''-CH), 141.4 (4'''a-C, 5a'-C), 144.1 (1'''a-C, 8'''a-C), 155.6, 156.2, 156.7 (N-CO-O-C(CH_3)$_3$ x 4, N-CO-O-CH_2 overlapping) 175.3 (O-CO-CH_2). FAB-MS *m/z* 1412 (MH^+), $C_{81}H_{130}N_6O_{14}$ requires 1410.



Attempted synthesis of ***N*¹-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(Oregon Green 488-amido)-pentanoic acid ester 82**

To a stirred solution of steroid **77** (6 mg, 4 μmol) in DMF (1 ml) was added KF (1 mg, 17 μmol) and TEA (1 ml, 7.7mmol) followed by Oregon Green 488 NSE (2 mg, 4 μmol). The solution was stirred at 25 °C under nitrogen for 10 h then evaporated to dryness *in vacuo* to give an orange oil. This oil was stirred in anhydrous CH_2Cl_2 (9 ml) and TFA (1 ml) at 25 °C for 3 h, then the solvent was evaporated *in vacuo* to afford a green oil which was purified by semi-preparative RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 488 nm, MeCN-0.01 % aq. TFA 9:1 v/v) to afford a green solid (11 mg, %). t_R 4.5 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 488 nm, MeCN-0.01 % aq. TFA 9:1 v/v). Absorption λ_{max} (H_2O): 489 nm. Fluorescence λ_{max} (Ex): 490 nm, λ_{max} (Em): 528 nm.

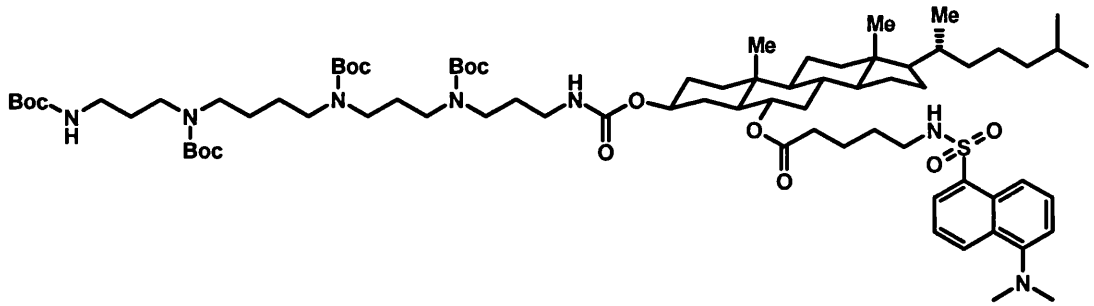


N^1-Dansyl-N^4,N^9,N^{12}-(tri-*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane 86

To a stirred solution of tri-Boc protected polyamine **5** (100 mg, 0.19 mmol) in CH_2Cl_2 was added TEA (1 ml, 7.7 mmol) and dansyl chloride **80** (54 mg, 0.19 mmol). The solution was stirred at 25 °C for 5 h then evaporated to dryness *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v) to afford the title compound as a green foam (88 mg, 63 %). R_f 0.82 (CH_2Cl_2-MeOH-conc. aq. NH_3 100:10:1 v/v/v). ^{1}H NMR ($CDCl_3$): 1.30-1.68 (35 H, m, 2-CH_2, 6-CH_2, 7-CH_2, 11-CH_2, O-C(C$\underline{H}_3$)$_3$ x 3), 2.88 (6 H, s, $(CH_3)_2N$), 2.97-3.26 (12 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 8-CH_2, 10-CH_2, 12-CH_2), 5.26-5.37 (1 H, m, CH_2-N$\underline{H}$-CO-O), 6.38-6.48 (1 H, m, N$\underline{H}$-SO_2), 7.17 (1 H, d, J = 7, 6'-CH), 7.49 (1 H, t, J = 7, 3'-CH), 7.56 (1 H, t, J = 7, 7'-CH), 8.21 (1 H, d, J = 7, 4'-CH), 8.37 (1 H, d, J = 7, 8'-CH), 8.50 (1 H, d, J = 7, 2'-CH). ^{13}C NMR: 25.8, 26.0 (6-CH_2, 7-CH_2), 28.3, 28.5 (2-CH_2, O-C(C$\underline{H}_3$)$_3$ x 3, overlapping), 37.3 (1-CH_2), 39.8 (12-CH_2), 43.7, 44.2 (3-CH_2, 10-CH_2), 45.4 ($(CH_3)_2N$), 46.8 (5-CH_2, 8-CH_2, overlapping), 79.4, 79.8, (O-C(C$\underline{H}_3$)$_3$ x 3, overlapping), 115.0 (6'-CH), 119.2 (8'-CH), 123.0 (3'-CH), 128.0 (4'-CH), 128.7 (7'-CH), 129.4, 129.6 (1'-C, 4a'-C), 129.7 (2'-CH), 135.5 (8a'-C), 151.5 (5'-C), 155.7, 156.1 (N-C$\underline{O}$-O-C$(CH_3)_3$ x 3). FAB-MS *m/z* 736 (MH^+), $C_{37}H_{61}N_5O_8S$ requires 735; FAB-HRMS calculated 736.4319 (MH^+), found 736.4321.

N^1-Dansyl-1,12-diamino-4,9-diazadodecane 87

Tri-Boc protected polyamine **86** (78 mg) in anhydrous CH_2Cl_2 (18 ml) and TFA (2 ml) was stirred at 25 °C for 3 h, then the solvent was evaporated *in vacuo*. The residue was washed with diethyl ether (3 x 10 ml) then recrystallised from EtOAc to afford the title compound as a buff solid (18 mg, 21 %). t_R 7.0 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 254 nm, MeCN-0.01 % aq. TFA 6:4 v/v). FAB-MS *m/z* 436 (MH^+), $C_{22}H_{37}N_5O_2S$ requires 435; FAB-HRMS calculated 436.2746 (MH^+), found 436.2734.



N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(6'α-hydroxy-5'α-cholesteryl-3'-oxycarbonyl)-1,16-diamino-4,8,13-triazahexadecane 5''-(dansylamido)-pentanoic acid ester 88

To a stirred solution of steroid **77** (7 mg, 5 μmol) in DMF (1 ml) was added KF (1 mg, 17 μmol) and TEA (1 ml, 7.7 mmol) followed by dansyl chloride (1 mg, 5 μmol). The solution was stirred at 25 °C under nitrogen for 3 h then diethyl ether (5 ml) and water (3 ml) was added. The organic layer was evaporated to dryness *in vacuo* to afford the title compound as a green wax. R_f 0.93 (EtOAc). FAB-MS *m/z* 1422 (MH^+), $C_{78}H_{131}N_7O_{14}S$ requires 1421; FAB-HRMS calculated 1422.9553 (MH^+), found 1422.9454.

N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)- 1,16-diamino-4,8,13-triazahexadecane hemisuccinate 4-(2-aminoethyl)-amino–*N*-octadecyl-1,8-naphthalimide conjugate tetratrifluoroacetate salt 93

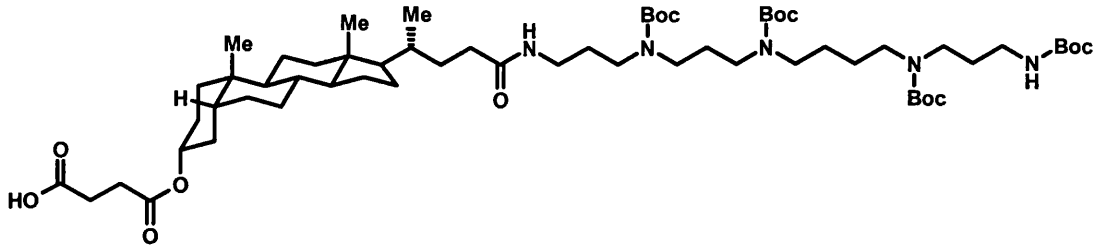
Tetra-Boc protected polyamine **97** (15 mg, 11 μmol) in anhydrous CH_2Cl_2 (9 ml) and TFA (1 ml) was stirred at 25 °C for 3 h. The solution was evaporated to dryness *in vacuo* and the residue purified by semi-preparative RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v) to afford the title compound as a yellow solid (11 mg, 71 %). t_R 5.2 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v). Absorption λ_{max} (H_2O): 453 nm. Fluorescence λ_{max} (Ex): 460 nm, λ_{max} (Em): 531 nm. FAB-MS *m/z* 1011 (MH^+), $C_{59}H_{94}N_8O_6$ requires 1010.

N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane hemisuccinate 4-(2-aminoethyl)-amino–*N*-octadecyl-1,8-naphthalimide conjugate tetratrifluoroacetate salt 94

Tetra-Boc protected polyamine **98** (12 mg, 7 μmol) in anhydrous CH_2Cl_2 (9 ml) and TFA (1 ml) was stirred at 25 °C for 3 h. The solution was evaporated to dryness *in vacuo* and the residue purified by semi-preparative RP-HPLC (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v) to afford the title compound as a yellow solid (8 mg, 64 %). t_R 5.4 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v). Absorption λ_{max} (H_2O): 439 nm. Fluorescence λ_{max} (Ex): 450 nm, λ_{max} (Em): 530 nm. FAB-MS *m/z* 1208 (MH^+), $C_{73}H_{122}N_8O_6$ requires 1207.

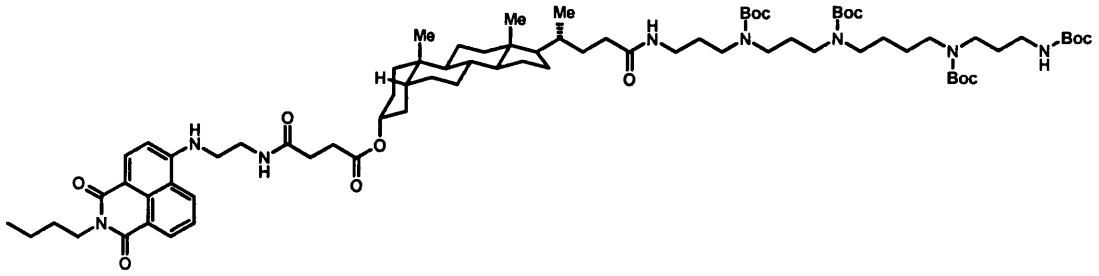
***N*-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1-aminopropane hemisuccinate 95**

Secondary alcohol **67** (400 mg, 0.96 mmol) and succinic anhydride (288 mg, 2.9 mmol) were stirred in hot pyridine (5 ml) at 110 °C for 20 h. Water (2 ml) was added, then the mixture was extracted with EtOAc (10 ml). The organic layer was washed with water (3 x 5 ml), dried ($MgSO_4$), and then evaporated *in vacuo*. The residue was recrystallised from EtOAc to afford the title compound as white crystals (416 mg, 84 %). ^1H NMR ($CDCl_3$): 0.64 (3 H, s, 18'-CH_3), 0.89-0.95 (9 H, m, 3-CH_3, 21'-CH_3, 19'-CH_3 overlapping), 0.96-2.30 (30 H, m, 2-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2), 2.56-2.68 (4 H, m, HOOC-C$\underline{H}_2$-C$\underline{H}_2$), 3.21 (2 H, q, *J* = 6, 1-CH_2), 4.67-4.79 (1 H, m, 3'-CH), 5.74-5.84 (1 H, m, NH-CO). ^{13}C NMR: 11.4 (N-CH_2-CH_2-$\underline{C}H_3$), 12.1 (18'-CH_3), 18.4 (21'-CH_3), 20.9 (11'-CH_2), 22.8 (N-CH_2-$\underline{C}H_2$-CH_3), 23.4 (19'-CH_3), 24.2 (15'-CH_2), 26.3, 26.6 (7'-CH_2 and HOOC-$\underline{C}H_2$-CH_2), 27.0 (6'-CH_2), 28.2 (16'-CH_2), 29.1, 29.5 (2'-CH_2 and CH_2-$\underline{C}H_2$-COO), 31.9 (22'-CH_2), 32.3 (4'-CH_2), 33.7 (23'-CH_2), 34.6 (10'-C), 35.0 (1'-CH_2), 35.5 (20'-CH), 35.8 (8'-CH_2), 40.1 (12'-CH_2), 40.4 (9'-CH), 41.3 (N-CH_2), 41.9 (5'-CH), 42.7 (13'-C), 56.0 (17'-CH), 56.4 (14'-CH), 74.8 (3'-CH), 171.6, 173.9 (CO-NH, O-$\underline{C}$O-CH_2), 176.3 (CO-OH).



N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane hemisuccinate 96

Secondary alcohol **50** (101 mg, 0.1 mmol) and succinic anhydride (50 mg, 0.5 mmol) were stirred in hot pyridine (5 ml) at 110 °C for 16 h. The reaction mixture was then concentrated *in vacuo*. The residue was purified over silica gel (CH_2Cl_2-MeOH 20:1 v/v) to afford the title compound as a white foam (93 mg, 84 %). R_f 0.25 (CH_2Cl_2-MeOH 20:1 v/v). ^{1}H NMR ($CDCl_3$): 0.64 (3 H, s, 18'-CH_3), 0.89-0.95 (6 H, m, 21'-CH_3 and 19'-CH_3 overlapping), 0.96-2.14 (74 H, m, 2-CH_2, 6-CH_2, 10-CH_2, 11-CH_2, 15-CH_2, 1'-CH_2, 2'-CH_2, 4'-CH_2, 5'-CH_2, 6'-CH_2, 7'-CH_2, 8'-CH, 9'-CH, 11'-CH_2, 12'-CH_2, 14'-CH, 15'-CH_2, 16'-CH_2, 17'-CH, 20'-CH, 22'-CH_2, 23'-CH_2, O-C(CH_3)$_3$ x 4), 2.55-2.68 (4 H, m, HOOC-C$\underline{H}_2$-C$\underline{H}_2$), 3.00-3.36 (16 H, m, 1-CH_2, 3-CH_2, 5-CH_2, 7-CH_2, 9-CH_2, 12-CH_2, 14-CH_2, 16-CH_2), 4.69-4.80 (1 H, m, 3'-CH), 5.28-5.42 (1 H, m, CH_2-N$\underline{H}$-CO-O), 6.76-6.93 (1 H, m, CH_2-N$\underline{H}$-CO-CH_2). ^{13}C NMR: 12.5 (18'-CH_3), 18.8 (21'-CH_3), 21.3 (11'-CH_2), 23.7 (19'-CH_3), 24.6 (15'-CH_2), 25.4, 26.5 (10-CH_2, 11-CH_2), 26.7, 27.0 (7'-CH_2 and HOOC-$\underline{C}H_2$-CH_2), 27.4 (6'-CH_2), 28.1, 28.1 (2-CH_2, 6-CH_2, 15-CH_2), 28.6 (16'-CH_2), 28.8, 28.9 (O-C($\underline{C}H_3$)$_3$ x 4), 29.5, 29.9 (2'-CH_2 and CH_2-$\underline{C}H_2$-COO), 32.2 (22'-CH_2), 32.6 (4-CH_2), 34.1 (23'-CH_2), 35.0 (10'-C), 35.4 (1'-CH_2), 35.9 (20'-CH), 36.2 (8'-CH), 37.8, 37.8 (1-CH_2, 16-CH_2), 40.5 (12'-CH_2), 40.8 (9'-CH), 42.3 (5'-CH), 43.1 (13'-C), 45.1, 45.1 (3-CH_2, 5-CH_2, 7-CH_2, 14-CH_2), 56.4 (17'-CH), 56.8 (14'-CH), 75.1 (3'-CH), 79.8 (O-$\underline{C}$(CH_3)$_3$ x 4), 172.0, 173.0 (CO-NH, O-$\underline{C}$O-CH_2), 176.3 (CO-OH acid). FAB-MS *m/z* 1119 (MH^+), 1117 ($M-H^+$)$^-$, $C_{61}H_{107}N_5O_{13}$ requires 1117; FAB-HRMS calculated 1118.7944 (MH^+), found 1118.7982.



N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane hemisuccinate 4-(2-aminoethyl)-amino–*N*-butyl-1,8-naphthalimide conjugate 97

EDC (8 mg, 36 μmol) and acid **96** (20 mg, 18 μmol) were dissolved in anhydrous CH_2Cl_2 (5 ml) and HOBt (1 mg, 7 μmol, 41 % cat) was added with stirring at 25 °C under nitrogen. Amine **91** (6 mg, 19 μmol) was added and the solution stirred for 16 h. The solution was evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 20:1 v/v) to afford the title compound as a yellow foam (21 mg, 75%). R_f 0.62 (CH_2Cl_2-MeOH 10:1 v/v). t_R 19.5 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v). FAB-MS *m/z* 1412 (MH^+), $C_{79}H_{126}N_8O_{14}$ requires 1411; FAB-HRMS calculated 1411.9472 (MH^+), found 1411.9495.

N^4,N^8,N^{13},N^{16}-(Tetra-*tert*-butyloxycarbonyl)-N^1-(3'α-hydroxy-5'β-cholan-24'-carbonyl)-1,16-diamino-4,8,13-triazahexadecane hemisuccinate 4-(2-aminoethyl)-amino–*N*-octadecyl-1,8-naphthalimide conjugate 98

EDC (6 mg, 32 μmol) and acid **96** (18 mg, 16 μmol) were dissolved in anhydrous CH_2Cl_2 (5 ml) and HOBt (1 mg, 7 μmol, 46 % cat) was added with stirring at 25 °C under nitrogen. Amine **92** (8 mg, 16 μmol) was added and the solution stirred for 10 h. The solution was evaporated to dryness *in vacuo* and the residue purified over silica gel (CH_2Cl_2-MeOH 20:1 v/v) to afford the title compound as a yellow foam (20 mg, 63%). R_f 0.80 (CH_2Cl_2-MeOH 10:1 v/v). t_R 21.8 min (Supelcosil ABZ+Plus, 5 μm, 25 cm x 10 mm, λ = 430 nm, MeCN-0.01 % aq. TFA 9:1 v/v). FAB-MS *m/z* 1608 (MH^+), $C_{93}H_{154}N_8O_{14}$ requires 1607; FAB-HRMS calculated 1608.1663 (MH^+), found 1608.1648.

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APPENDIX

Publications and abstracts from this work

The following title is in press:

"Design and Synthesis of Fluorescent Steroidal Lipopolyamine Conjugates for Monitoring Gene Delivery", in **Bioactive Drugs in Drug Discovery and Design: Medical Aspects**, IOP Press: Amsterdam

The following titles have been presented as poster or oral presentations:

"Fluorescent Steroidal Lipopolyamine Conjugates for Monitoring Gene Delivery", Oral
RSC Bio-Organic Symposium, University of East Anglia (17.12.01),
British Pharmaceutical Conference, Glasgow (24.09.01), *J. Pharm. Pharmacol.*, **2001**, *53*, S1
221st American Chemical Society National Conference, San Diego, USA (05.04.01), *Abs. Pap. Am. Chem. Soc.*, **2001**, *221*, 352-MEDI, Part 2
School of Pharmacy, University of Southern California, Los Angeles, USA (30.03.01)

"Synthesis and Spectroscopic Studies of Fluorescent Lipopolyamine-DNA Complexes", Oral
RSC Bio-Organic Symposium, University of Warwick (18.12.00)

"Design and Synthesis of Fluorescent Cholesterol and Lithocholic Acid Polyamine Conjugates", Poster
221st American Chemical Society National Conference, San Diego (04.04.01), *Abs. Pap. Am. Chem. Soc.*, **2001**, *221*, 332-MEDI, Part 2
RSC East of England Medicinal Chemistry Symposium, University of Hertfordshire (26.04.01)
British Pharmaceutical Conference, Glasgow (26.09.01), *J. Pharm. Pharmacol.*, **2001**, *53*, S245

"Hydroboration of Cholesterol Carbamates: Novel Fluorescent Lipopolyamines", Poster
British Pharmaceutical Conference, Birmingham (10.09.00), *J. Pharm. Pharmacol.*, **2000**, *52*, S116

Design and Synthesis of Fluorescent Steroidal Lipopolyamine Conjugates for Monitoring Gene Delivery

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Design and Synthesis of Fluorescent Steroidal Lipopolyamine Conjugates for Monitoring Gene Delivery

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Abstract: The rational design and practical preparation of unsymmetrical polyamine carbamates and amides, based on steroid templates of cholesterol and the bile acid lithocholic acid as the lipid moiety, provides fluorescent molecular probes that condense DNA. These novel lipopolyamine conjugates mimic the positive charge distribution found in the triamine spermidine and the tetra-amine spermine alkaloids. These probes are useful in monitoring gene delivery for non-viral gene therapy.

1. Introduction and Background

Gene delivery for gene therapy of a variety of human and also for animal diseases e.g. cystic fibrosis [1], hepatitis [2], haemophilia [3], cancer [4-6], and more speculatively mitochondrial gene therapy for respiratory chain defects, i.e. defects that involve the final common pathway of oxidative metabolism [7], can be performed with either a viral [8-10] or a non-viral vector [11-14]. Lipopolyamines (cationic lipids) recently developed for non-viral gene therapy show significantly lower toxicity than viral vectors, although they are currently less efficient transfection vectors than viruses [11-14]. The poor efficiency of DNA delivery to the nucleus, especially using non-viral vectors, is a major limitation of the gene therapy approach. A poor understanding of the molecular mechanisms of action in non-viral gene delivery [11, 15], and a lack of correlation between *in vivo* and *in vitro* biological activity [16-18] are unresolved issues and constitute the main current challenges. Whilst true gene therapy, i.e. the expression in cells of genetic material that has therapeutic activity, holds promise for the treatment of the significant human diseases listed above, the non-viral gene delivery vehicle, or vector, carrying the genetic material into the cell, must be optimised to increase the efficiency of gene delivery to eukaryotic cells. Uptake of condensed DNA by target cells proceeds via endocytosis, but poor nuclear localisation is another major barrier to efficient gene expression (measured using reporter genes e.g. β-galactosidase expression). Thus, a greater knowledge of the molecular mechanisms of transfection will provide a basis for rational non-viral vector design [11-15, 19-24]. The possibility of also enhancing the efficiency of non-viral gene delivery to the nucleus by incorporating specific nuclear targeting (localisation) sequences in vectors is another area of interest [23, 24]. Therefore, our aims in this research programme include the design and synthesis of useful molecular probes, initially as model compounds and then for the study of DNA-lipopolyamine vector interactions at the molecular and intracellular levels.

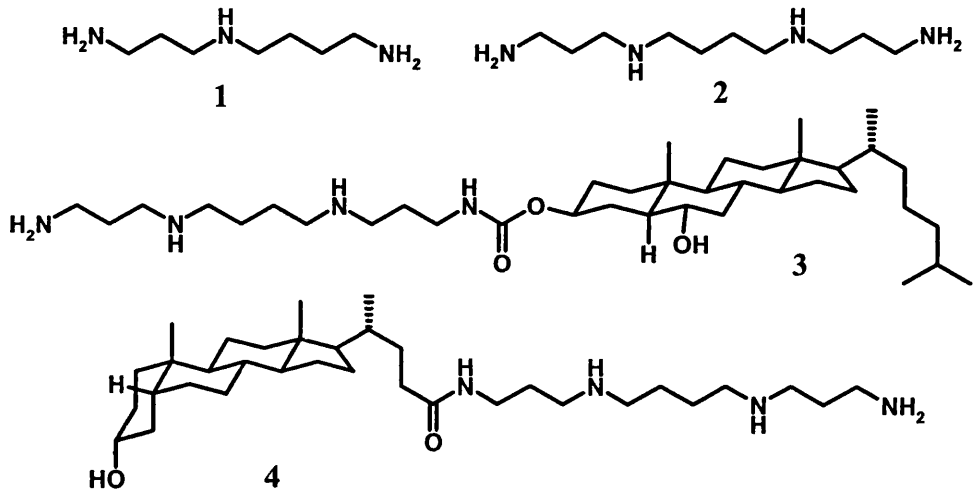

Figure 1

Polyamines are polycationic at physiological pH and play key roles in biological systems. In chromatin, polyamines such as the triamine spermidine **1** and tetra-amine spermine **2** (Fig. 1) help to package DNA into nuclei through neutralisation of the poly-anionic phosphate backbone charges, bringing about DNA condensation and assisting in the control of DNA solution conformations. Lipopolyamines condense DNA more efficiently than simple polyamines [25] - this is a key first step in non-viral gene delivery. Employing a high molecular weight cationic polymer for non-viral gene delivery to transfect target cells, is often associated with unacceptably high toxicity [26]. Small molecule lipopolyamines are therefore of considerable interest due to their advantages of low toxicity, low immunogenicity, controllable syntheses, and defined molecular structure for pharmaceutical characterisation. We are studying the synthesis of steroidal polyamine conjugates, secondary alcohol functionalised cholesteryl carbamate **3** and lithocholic acid amide **4** (Fig. 1). Lipopolyamines reported in this research area (Fig. 2) include *inter alia*: Transfectam **5** [27] and RPR-120535 **6** [28] have branched or linear spermine moieties linked to two C_{18}-lipid chains, whereas Genzyme's Lipid GL #67 **7** possesses a cholesteryl lipid moiety [17]. Vitamin D_2 (ergocalciferol) polyamine conjugate **8** is a steroid with an open B-ring, designed to probe the structure-activity effects on transfection of modifying the geometry within the hydrophobic steroid motif [29]. Buckminster Fullerene tetra-ammonium ion conjugate **9** is typical of a new class of cationic lipid incorporating a C_{60} hydrophobic core, where the amine moiety was conjugated to fullerene by a cycloaddition reaction [30]. Gemini surfactant GS4 **10** is a tetra-lysine based cationic lipid, carrying a maximum of 6 positive charges and conjugated with two C_{12}-lipid chains [31, 32]. Novel spermine-based cationic gemini surfactants symmetrically acylated with two C_{18}-lipid and two tri-lysine chains, with up to 8 positive charges, have also recently been reported [33].

Non-viral transfection efficiency cannot rationally be improved without a greater knowledge of the detail of intracellular events. In order to address the rational design of small molecule vectors, the molecular mechanisms of these poorly understood steps must be delineated. Fluorescent microscopy is useful for the study of intracellular events. We are therefore synthesising the functionalised steroid polyamine conjugates cholesteryl carbamate **3** and lithocholic acid amide **4** (Fig. 1) as DNA condensing agents which we have designed in order that a variety of fluorophores can be introduced allowing the rapid synthesis of a required library of steroidal lipopolyamine fluorescent probes [34-38]. The polyamine conjugates are designed to mimic the charge distribution found in the naturally occurring triamine spermidine **1**, and are prepared by regio-controlled conjugation of a suitably protected derivative **11** of the symmetrical polyamine spermine **2** (Fig. 3) [39, 40].

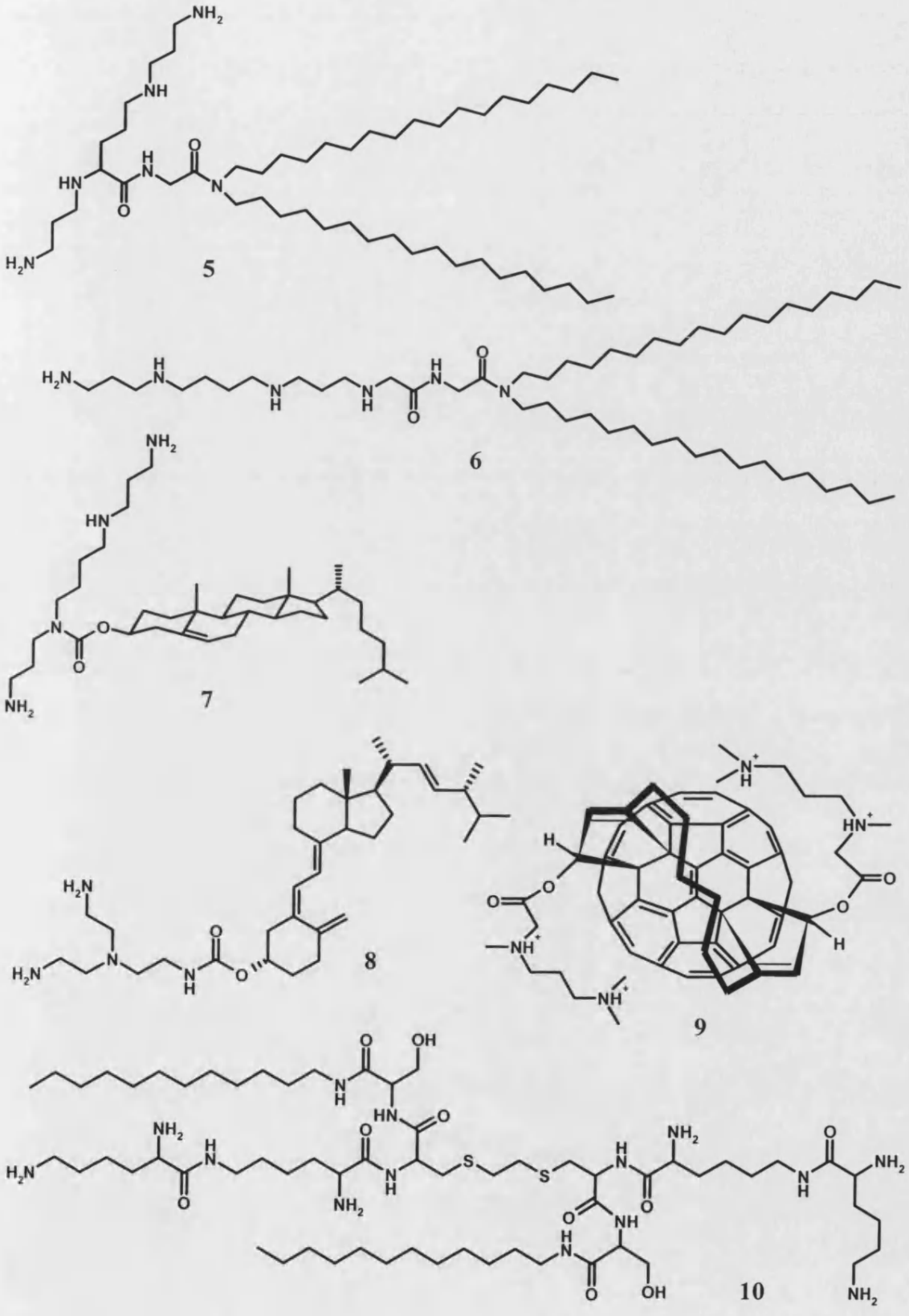

Figure 2

2. Results and Discussion

2.1 Design and Synthesis of Functionalised Polyamine-Steroid Conjugates

The synthesis of the small molecule ligands **3** and **4** is described in outline. Spermine **2** was unsymmetrically tri-*tert*-Boc protected in a one-pot reaction (Fig. 3) [39, 40]. Ethyl trifluoroacetate reacts rapidly and cleanly with primary amines, allowing poly-Boc protection of all the remaining amine functional groups. Trifluoroacetamides are easily cleaved, in the presence of Boc groups, at pH 11 and treatment with conc. aqueous NH_3 gave one free primary amine functional group in 50% overall yield. Acylation with cholesteryl chloroformate gave the protected cholesteryl carbamate **12** in 95% yield [40].

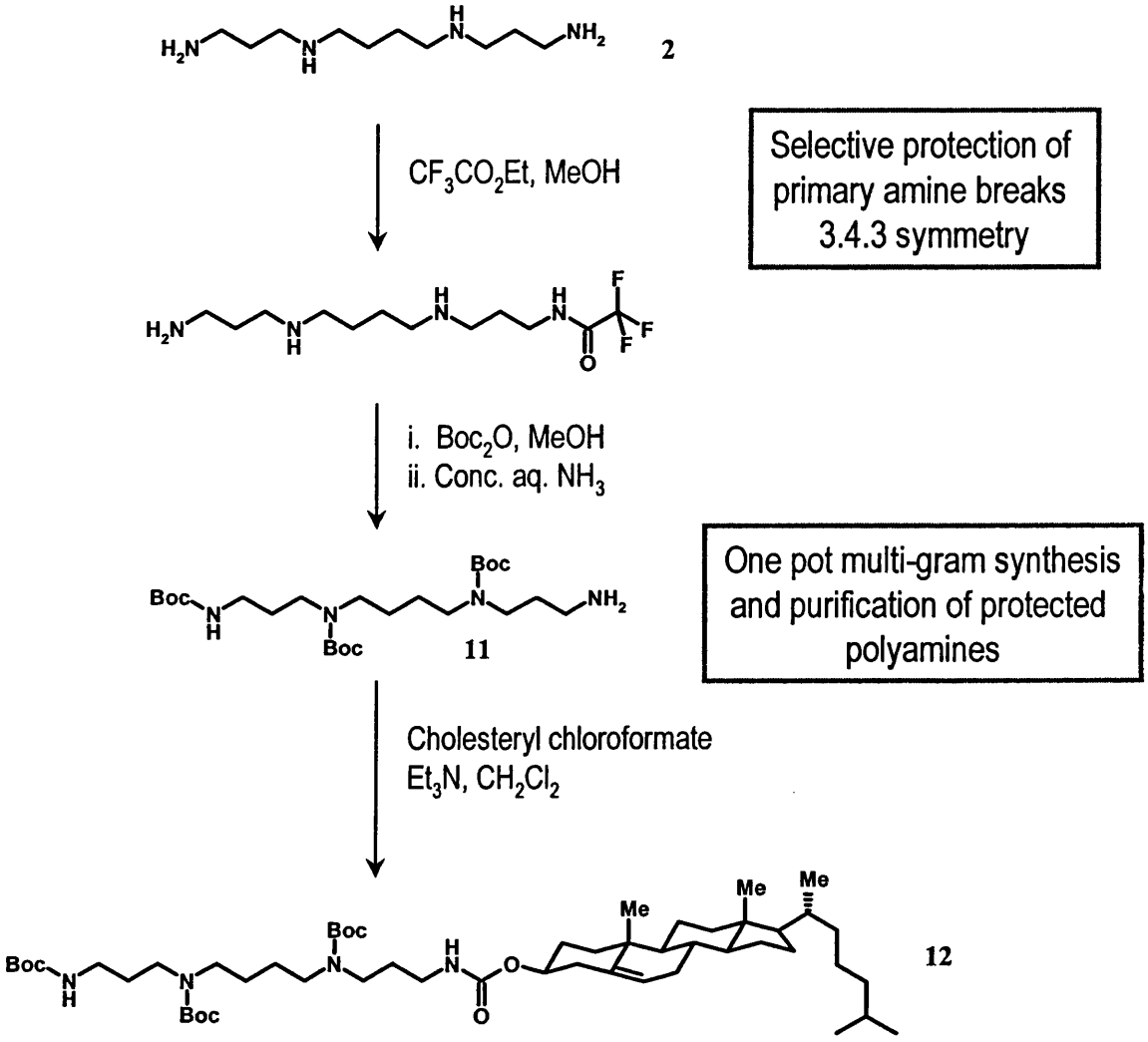

Figure 3

For fluorescent microscopy to study the intracellular phenomena of non-viral gene delivery, Byk *et al.* reported a fluorescent derivative of a polyamine conjugated to two C_{18}-lipid chains and they have synthesised fluorescent plasmid DNA using rhodamine-type fluorophores [28, 41]. Polyethylenimine (PEI) is a cationic aziridine polymer with known transfection activity [42]. Mikos and co-workers used Oregon Green labelled PEI in multicolour fluorescence studies and provided initial evidence that internalised DNA-PEI complex can be tracked to the nucleus [43]. We have set ourselves the goal of lighting-up the inside of the cell with wavelength-specific small molecule fluorescent probes and monitoring the processes in, and elucidating the barriers to efficient gene delivery.

For the introduction of a fluorescent label to cholesteryl, we decided to hydroborate the cholesterol Δ^5-alkene functional group [44]. Tri-Boc protected cholesteryl carbamate **12** was reacted with borane-dimethyl sulfide (BH_3.dms) for 4 h in anhydrous CH_2Cl_2. The organoborane was oxidised for 30 min with basic ($NaHCO_3$) H_2O_2. The use of BH_3.dms complex provides mild conditions for the selective reaction of an alkene in the presence of carbamate functional groups, despite the poor reactivity of the cholesteryl alkene due to a significant steric constraint. The methyl substituents on the top face (β-face) of cholesteryl direct the *syn*-addition of borane to the α-face in the major product. The desired 6α-hydroxy steroid **13**, with a *trans*-fused AB ring junction, is obtained on oxidative work-up and after chromatography in 70% yield. In addition, a small fraction of the BH_3.dms adds to the β-face affording the *cis*-fused AB, 6β-OH stereoisomer **14** (5%) which is more polar and these products were separated by column chromatography over silica gel. Given that hydroboration is a *syn*-addition, in the less-polar major product H-6_{ax} displays two $J_{ax\text{-}ax}$ and one $J_{ax\text{-}eq}$ coupling at δ 3.35 ppm, dt (J = 5 and 11 Hz) (Fig. 4), R_f 0.5, EtOAc-hexane 1:1 v/v, ^{13}C NMR: δ 52.3 (C-5), 68.4 (C-6), 73.9 (C-3) ppm, HR FAB-MS found 933.7321 (M^+ + 1), $C_{53}H_{97}N_4O_9$ requires 933.7256, was the desired *trans*-fused AB system. On further elution, a minor product was obtained, R_f 0.1, EtOAc-hexane 1:1 v/v, HR FAB-MS found 933.7274 (M^+ + 1), this was assigned to the β-hydroxy isomer from a non-stereospecific reaction [45]. The *cis*-fused AB ring junction, in the minor product **14**, mimics the shape found in the naturally occurring steroid bile acids (Fig.5), e.g. lithocholic acid, polyamine amides of which, e.g. **4** we have shown to condense duplex DNA [46, 47].

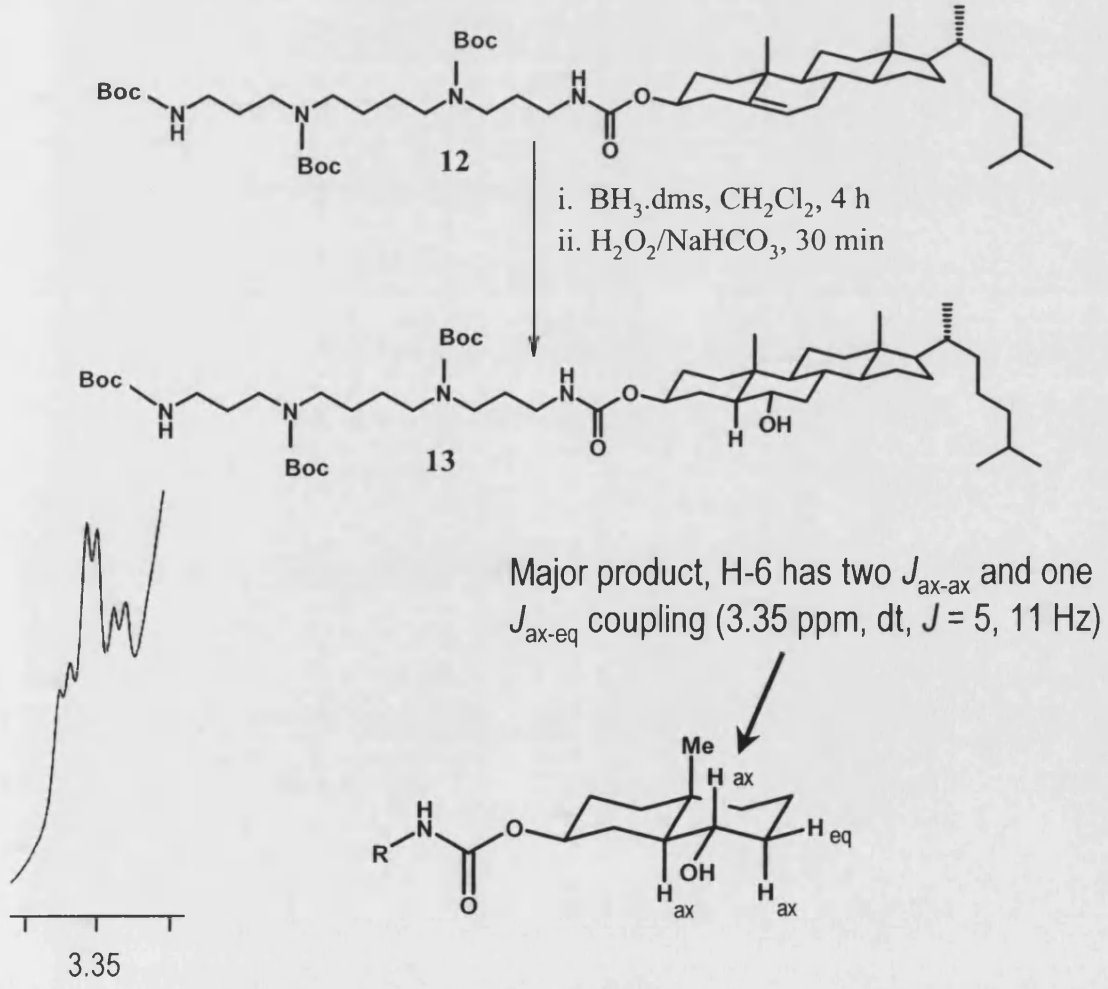

Figure 4

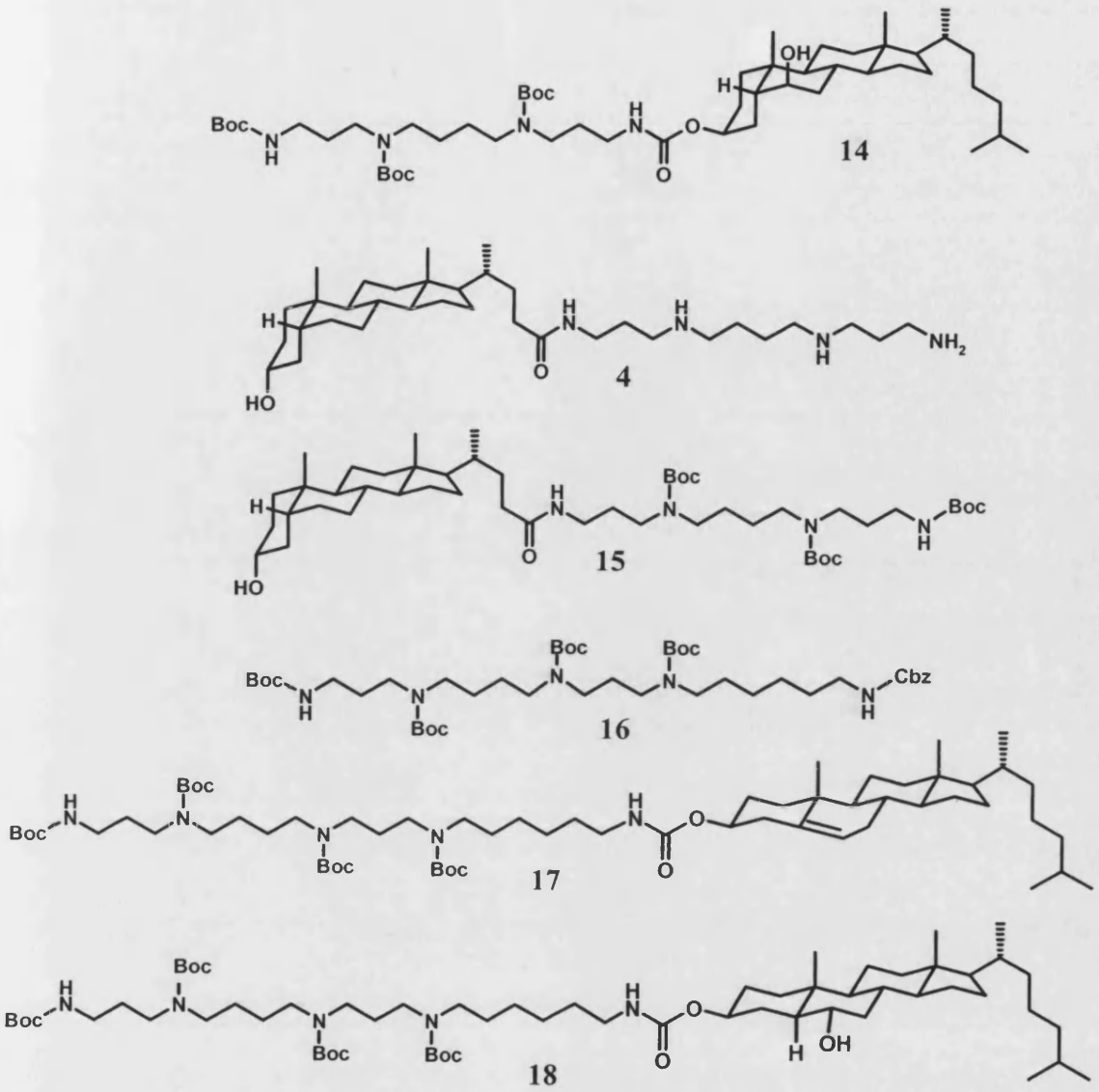

Figure 5

Similarly, we prepared spermidine mimic target molecule **4** from TFA deprotection of the three Boc protecting groups in **15**, itself obtained by acylation of amine **11** with lithocholic acid (DCC, HOBt, CH_2Cl_2). In order to mimic the positive charge distribution of naturally occurring spermine **2** in the target molecules, we have investigated a reductive alkylation approach using N^1,N^2,N^3-tri-Boc spermine **11**. *N*-Cbz protected 6-aminohexan-1-ol was prepared using benzyl chloroformate. The alcohol was oxidised under Swern conditions (anhydrous DMSO-oxalyl chloride) to the corresponding aldehyde followed by reductive amination with N^1,N^2,N^3-tri-Boc spermine **11** [39, 40]. The resulting secondary amine was Boc protected to form tetra-Boc-mono-Cbz pentamine with methylene spacing 3.4.3.6 **16**. The Z group, orthogonal to Boc, was then cleaved by palladium catalysed hydrogenolysis or by transfer hydrogenation using cyclohexene as a H_2-donor. These steps achieved an unsymmetrical extension of N^1,N^2,N^3-tri-Boc spermine **11** with assured regiochemistry in the product **17** after acylation with cholesteryl chloroformate. The resulting tetra-Boc protected 3.4.3.6-cholesteryl carbamate **17** was hydroborated using the procedure outlined above and the major product was isolated, purified, and identified as secondary alcohol **18** FAB-MS found 1154 ($M + Na^+$), $C_{64}H_{117}N_5O_{11}$ requires 1131.

Fluorescent labels were then introduced to these lipopolyamine conjugates to generate our designed fluorescent target molecules. Fmoc, as either a fluorescent tag or as a primary amine protecting group, was first introduced by esterification (DCC, DMAP) with the short alkyl chain 5-Fmoc-aminopentanoic acid (δAPA), chosen as an appropriate and pragmatic spacer. The secondary alcohols of cholestane B-ring **13** affording ester **19**, and of lithocholic acid A-ring **15** affording **20** were reacted in this manner (Fig. 6). Further manipulation of these esters **19** and **20** by regioselective deprotection of one primary amine functional group by fluoride facilitated Fmoc removal with TBAF [48], and then reaction with fluorescein isothiocyanate afforded, after TFA removal of the Boc protecting groups, thiourea target molecules **21** and **22** respectively. It is significant that the secondary alkyl cholesteryl carbamate functional group remains intact during this poly-Boc deprotection. Final purification of these water soluble poly-trifluoroacetic acid salts afforded the desired fluorescent steroidal lipopolyamine conjugates which can react with DNA.

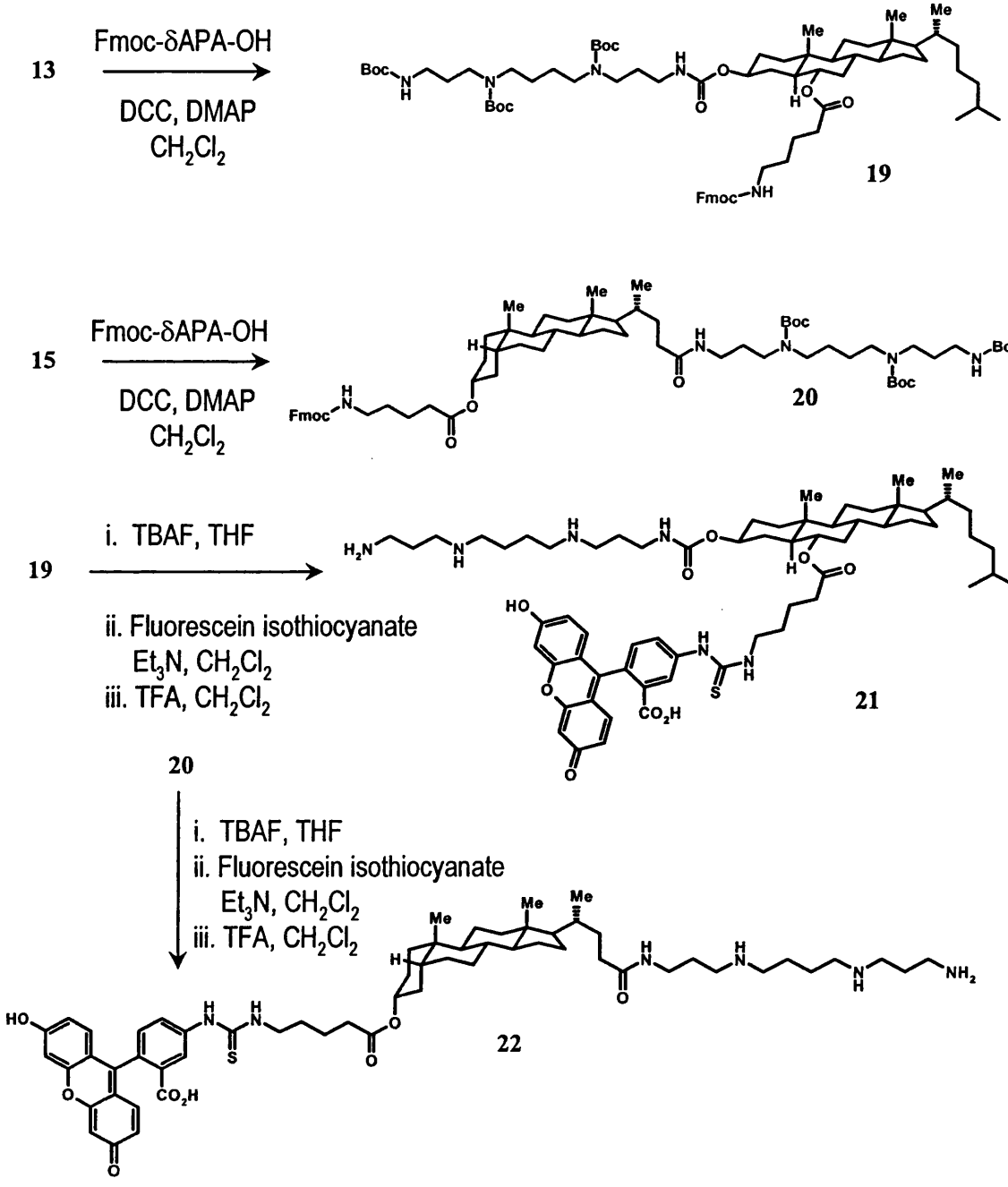

Figure 6

2.2 Monitoring Lipopolyamine Interactions with DNA

Spectroscopic assays allow us to monitor DNA condensation, the first stage in lipopolyamine mediated gene delivery, using our designed probes **3**, **4**, **21**, and **22** (Fig. 7). These DNA assays include both ethidium bromide fluorescence quenching [49] and light scattering (λ = 320 nm) [50] as, at this stage, the DNA is compacted into toroidal particles whose formation can be monitored by light scattering [50]. Using these techniques, we have shown that our synthetic steroidal lipopolyamines e.g. **4** and cholesteryl carbamate **23** from Boc-deprotection of **12** [40], an N^1-acylated regioisomer of GL #67 **7** [17], condense DNA more efficiently than the natural polyamines spermidine **1** and spermine **2**. The synthetic lithocholic acid amide of spermine **2**, converted into an ester and then a thiourea with fluorescein isothiocyanate, spermidine equivalent **22**, condensed both calf thymus and synthetic DNA with the same efficiency as spermidine **1** in the ethidium bromide assay.

3. Conclusions and Future Studies

Using our designed steroidal lipopolyamine probes (Fig. 7), we are studying DNA-lipopolyamine complexes with respect to their formation by DNA condensation. The design and synthesis of fluorescent lipopolyamines allows us to study the intracellular events during transfection. Comparable DNA-binding efficiency to that of the unlabelled lipopolyamines and robust fluorescent spectral properties across the varying cellular pH range are desirable properties in these ligands. We have achieved a controlled chain extension of suitably protected polyamines using reductive alkylation [39] to mimic spermine **2** rather than spermidine **1** in the target molecules. A practical method for the efficient hydroboration of cholesteryl carbamates has allowed us to prepare our designed *trans*-AB steroidal lipopolyamines, together with the corresponding *cis*-AB ring junction as the minor product of the hydroboration reaction or from naturally occurring bile acids. We are able to introduce fluorophores of choice by our Fmoc-chemistry. In this way, we have synthesised the beginnings of a library of fluorescent lipopolyamine probes based on steroid moieties as the lipophilic unit. The design in our versatile synthetic route to Fmoc protected aminoesters of cholesteryl carbamate **19** and lithocholic acid polyamine amide **20** allows a range of selected fluorophores to be readily incorporated e.g. fluorescein isothiocyanate which is a widely used fluorescent probe in confocal microscopy (cf **3** and **21**, and **4** and **22**). The availability of a wide range of fluorophores is important in order that a library of fluorescent derivatives may be accessed via this Fmoc deprotection route. *N*-Hydroxysuccinimide activated reagents to introduce these fluorophores include Oregon Green 488 **24** (Fig. 7), a difluorinated fluorescein analogue which offers improved spectral properties over the physiological pH range involved in transfection processes and Alexa Fluor 350 **25**. Together with fluorescein, fluorophores **24** and **25** are negatively charged. Lissamine Rhodamine B **26** and other rhodamine-type fluorophores incorporate positive charges; Fmoc **27** and BODIPY-FL **28** fluorophores are lipophilic. Steroid lipopolyamine conjugates of these fluorophores will have different efficiencies in the modes of conjugate binding to DNA. As well as our studies on lipopolyamine-mediated DNA condensation, we are optimising these probes to use them in monitoring the steps of gene delivery within cells. At present, a poor understanding of the mechanisms of action of non-viral vectors remains an important unresolved issue. With a greater knowledge of these mechanisms [51, 52], new non-viral vectors with improved transfection efficiency can be rationally designed. In conclusion, these results help us to design more efficient molecular probes for DNA-binding in this area of non-viral gene delivery using lipopolyamine moieties.

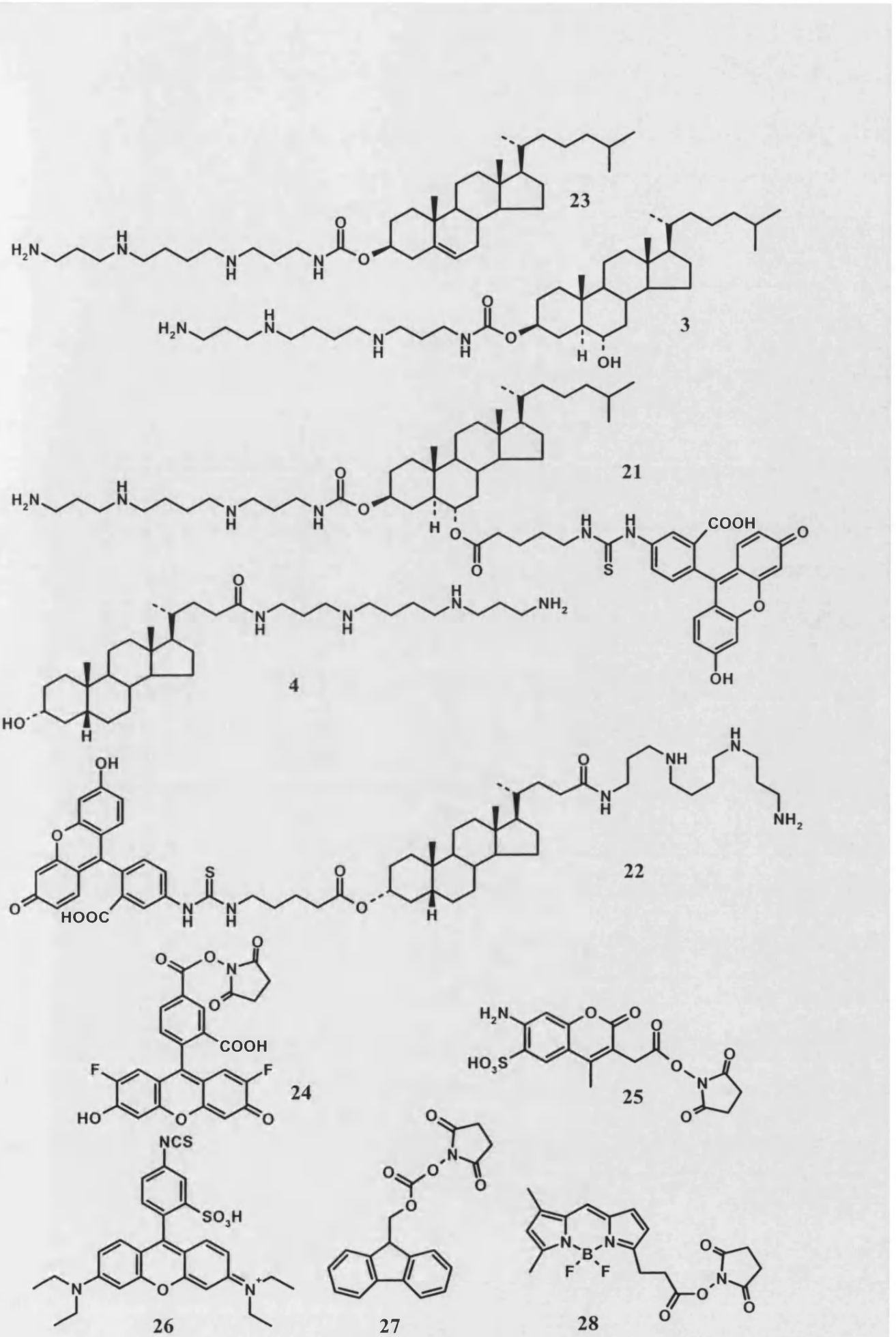

Figure 7

4. Acknowledgements

We acknowledge financial support from the EPSRC (studentship to A.P.N.). The results in these studies in synthetic and analytical medicinal chemistry, related to "Nucleic Acids – Ligands for Gene Therapy", are in part based upon our recent oral and poster presentations, published in various abstracts [34-38].

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Hydroboration of Cholesterol Carbamates: Novel Fluorescent Lipopolyamines

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Lipopolyamines mimic naturally occurring polyamines in the condensation of DNA and have recently been developed as potential DNA delivery vectors for use in non-viral gene therapy. Currently, the mechanisms of lipopolyamine mediated transfection are poorly understood. This inefficiency cannot be resolved without a greater knowledge, and efforts to study intracellular events during non-viral transfection are underway. These efforts are focused around the use of fluorescent microscopy Byk et al (1998), Helbling-Leclerc et al (1999) and Godbey et al (1999). RPR-121653 is a fluorescent polyamine conjugated to two C18 lipid chains, Byk et al (1998). Polyamine carbamates of cholesterol are currently the lead compounds in this area of non-viral transfection. Therefore, we have designed the first fluorescent steroidal polyamine (1).

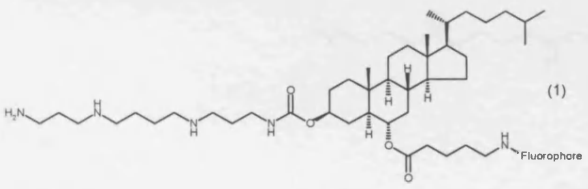

Figure 1. Target fluorescent polyamine-cholesterol carbamate (1)

The key step in the functionalisation of cholesterol is hydroboration followed by oxidative workup. The use of borane-dimethyl sulfide complex provides mild conditions for the selective treatment of an alkene versus carbamate functional groups, despite poor reactivity of the alkene of cholesterol due to a heavy steric constraint Tavares et al (1993). The *syn*-addition across the double bond of cholesterol furnishes the steroid with a 6α-hydroxy group and a *trans*-fused A-B ring junction (2). This step has been achieved in 70 % yield, observed key ^{13}C NMR values (starting material in brackets) include: 52.3 (140.0) (C-5); 68.4 (122.3) (C-6); 73.9 (74.1) (C-3) and FAB-MS found 956 ($M^+ + Na^+$), $C_{53}H_{96}N_4O_9$ requires 933).

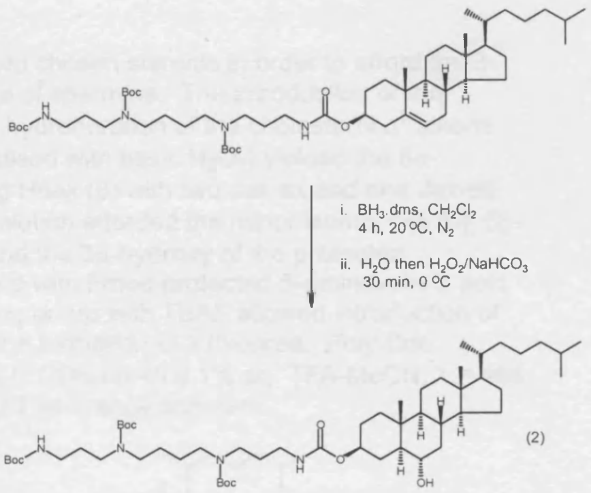

Figure 2. Introduction of 6α-hydroxyl onto a polyamine-cholesterol carbamate (2)

The functional group modification of the steroid will allow derivatisation of a lipopolyamine with a fluorophore of choice, via a short chain ester or ether. The synthesis concludes with poly-Boc deprotection in TFA-CH_2Cl_2 and purification by RP-HPLC.

We thank the EPSRC for a studentship to A.P.N.

1. Byk G., Dubertret-C., Escriou V., Frederic M., Jaslin G., Rangara R., Pitard B., Crouzet J., Wils P., Schwartz B., Scherman D. (1998) J. Med. Chem., 41: 224-235
2. Helbling-Leclerc A., Scherman D, Wils P. (1999) Biochim. Biophys. Acta, 1418: 165-175
3. Godbey W. T., Wu K. K., Mikos A. G. (1999) Proc. Natl. Acad. Sci. USA, 96: 5177-5181
4. Tavares R., Randoux T., Braekman J.-C. and Daloze D. (1993) Tetrahedron, 49: 5079-5090

Abstract submission for poster session in Medicinal Chemistry

APN Oral Abstract for Warwick RSC Bio-Organic

Synthesis and Spectroscopic Studies of Fluorescent Lipopolyamine-DNA Complexes

Adrian P. Neal and Ian S. Blagbrough
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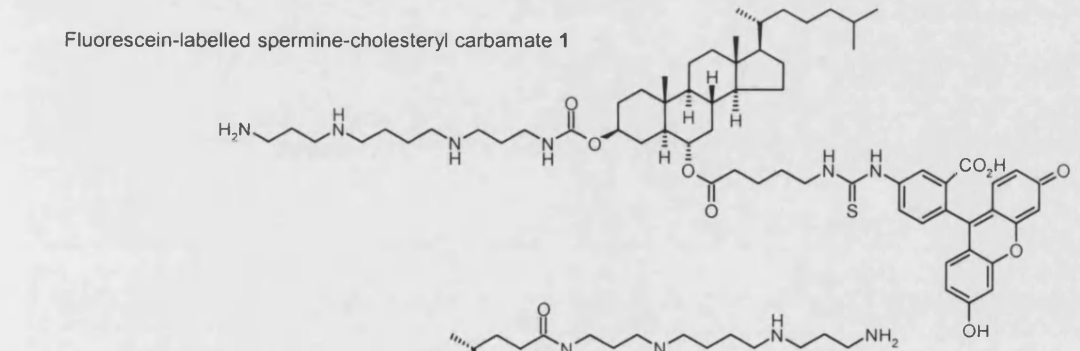
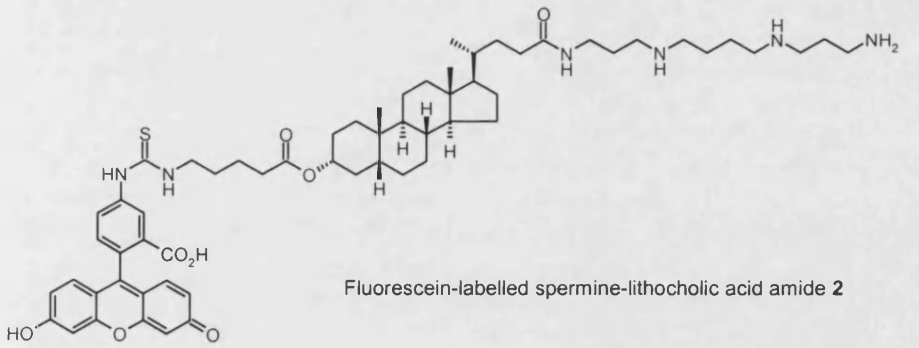
The rational design of efficient non-viral gene delivery vectors is currently restricted by a poor understanding of the molecular detail of intracellular events. As fluorescent microscopy is potentially useful for the study of lipopolyamine-mediated transfection, the design of fluorescent probes is required. We are studying steroidal polyamine conjugates, cholesteryl carbamates and lithocholic acid amides as efficient DNA condensing agents, that have been designed in order that a variety of fluorophores may be introduced allowing rapid synthesis of a library of fluorescent lipopolyamines (with both *cis*- and *trans*-A-B steroids). The synthesis of these novel fluorescent probes is described here.

Tri-Boc-protected spermine was acylated with the two chosen steroids in order to afford the 3-cholesteryl carbamate and the lithocholic acid amide of spermine. The introduction of the fluorescent label on cholesteryl carbamate requires hydroboration of the cholesterol Δ^5 alkene functional group. *syn*-Hydroboration (BH_3.dms, oxidised with basic H_2O_2) yielded the 6α-hydroxy steroid, a *trans*-A-B ring junction, displaying H6ax (β) with two *J*ax-ax and one *J*ax-eq at δ = 3.35 ppm, (dt, *J* = 4.5 and 10.9 Hz). Further elution afforded the minor isomer (~5 %), 6β-hydroxy, a *cis*-A-B ring junction. This 6α-hydroxy and the 3α-hydroxy of the protected lithocholic acid polyamine amide have been esterified with Fmoc-protected 5-aminovaleric acid (DCC/DMAP). Facile removal of the Fmoc-protecting group with TBAF allowed introduction of the fluorophore fluorescein isothiocyanate through the formation of a thiourea. Poly-Boc deprotection (TFA/CH_2Cl_2) and purification (RP-HPLC ODS 60:40 0.1% aq. TFA-MeCN, λ = 495 nm) afforded the target polyamine conjugates **1** and **2** as orange powders.

Fluorescein-labelled spermine-cholesteryl carbamate **1**





Fluorescein-labelled spermine-lithocholic acid amide **2**

Spectroscopic assays exist for monitoring DNA condensation using such designed probes. These include a fluorescence quenching ethidium bromide assay and a light scattering (λ = 320 nm) assay. Using these techniques, we have shown that our synthetic lipopolyamines condense DNA more efficiently than the natural polyamines spermidine and spermine. A synthetic model lithocholic acid amide of spermine (a spermidine equivalent) derivatised with fluorescein isothiocyanate condensed both calf thymus and synthetic DNA with the same efficiency as spermidine in the ethidium bromide assay. These results help us to design more efficient fluorescent probes for DNA-binding in this area of non-viral gene delivery using alkyl spermine moieties. We acknowledge financial support from the EPSRC (studentship to A.P.N.).

APN Poster Abstract for San Diego ACS

Design and synthesis of fluorescent cholesterol and lithocholic acid polyamine conjugates

Adrian P. Neal and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

Lipopolyamines are lead compounds for efficient DNA delivery vectors in non-viral gene therapy. 3-Cholesteryl polyamine carbamates and lithocholic acid polyamine amides condense DNA, therefore we have designed fluorescent polyamine probes incorporating these steroid moieties. The synthesis of these novel fluorescent lipopolyamines, with both *trans*- and *cis*-AB steroid polyamines, is described.

Tri-Boc-protected spermine was acylated with the two chosen steroids in order to afford the 3-cholesteryl carbamate and the lithocholic acid amide of spermine. The introduction of the fluorescent label on cholesteryl carbamate requires hydroboration of the cholesterol Δ^5 alkene functional group. *syn*-Hydroboration (BH_3.dms, oxidised with basic H_2O_2) yielded the 6α-hydroxy steroid, a *trans*-A-B ring junction, displaying H6ax (β) with two *J*ax-ax and one *J*ax-eq at δ = 3.35 ppm, (dt, *J* = 4.5 and 10.9 Hz). Further elution afforded the minor isomer (~5 %), 6β-hydroxy, a *cis*-A-B ring junction. This 6α-hydroxy and the 3α-hydroxy of the protected lithocholic acid polyamine amide have been esterified with Fmoc-protected 5-aminovaleric acid (DCC/DMAP). Facile removal of the Fmoc-protecting group with TBAF allowed introduction of the fluorophore fluorescein isothiocyanate through the formation of a thiourea. Poly-Boc deprotection (TFA/CH_2Cl_2) and purification (RP-HPLC ODS 60:40 0.1% aq. TFA-MeCN, λ = 495 nm) afforded the target polyamine conjugates as orange powders.
We acknowledge financial support from the EPSRC (studentship to A.P.N.).

APN Oral Abstract for San Diego ACS

Fluorescent steroidal lipopolyamine conjugates for monitoring gene delivery

Adrian P. Neal and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

In non-viral gene therapy, the mechanisms of the intracellular steps are currently poorly understood. Transfection efficiency cannot be improved without a greater knowledge of the molecular detail of intracellular events. As fluorescent microscopy is potentially useful for the study of lipopolyamine-mediated transfection, the design of fluorescent probes is required. We are studying steroidal polyamine conjugates, cholesteryl carbamates and lithocholic acid amides, as efficient DNA condensing agents that have been designed in order that a variety of fluorophores may be introduced allowing rapid synthesis of a library of fluorescent lipopolyamines.

Spectroscopic assays exist for monitoring DNA condensation using such designed probes. These include a fluorescence quenching ethidium bromide assay and a light scattering (λ = 320 nm) assay. Using these techniques, we have shown that our synthetic lipopolyamines condense DNA more efficiently than the natural polyamines spermidine and spermine. A synthetic model lithocholic acid amide of spermine (a spermidine equivalent) derivatised with fluorescein isothiocyanate condensed both calf thymus and synthetic DNA with the same efficiency as spermidine in the ethidium bromide assay. These results help us to design more efficient fluorescent probes for DNA-binding in this area of non-viral gene delivery using alkyl spermine moieties.

We acknowledge financial support from the EPSRC (studentship to A.P.N.).

APN poster abstract for RSC East of England Med Chem Symposium 26.04.01

Design and synthesis of fluorescent cholesterol and lithocholic acid polyamine conjugates

Adrian P. Neal and Ian S. Blagbrough

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

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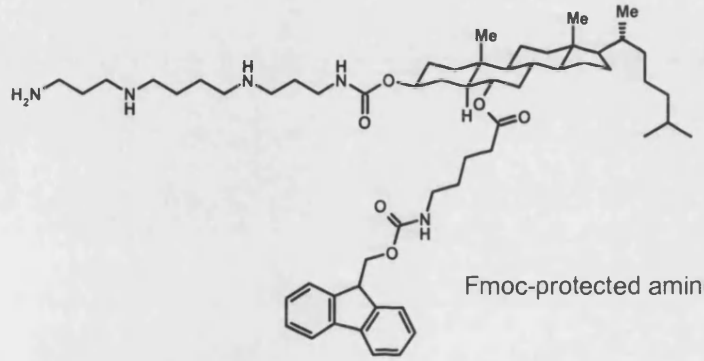
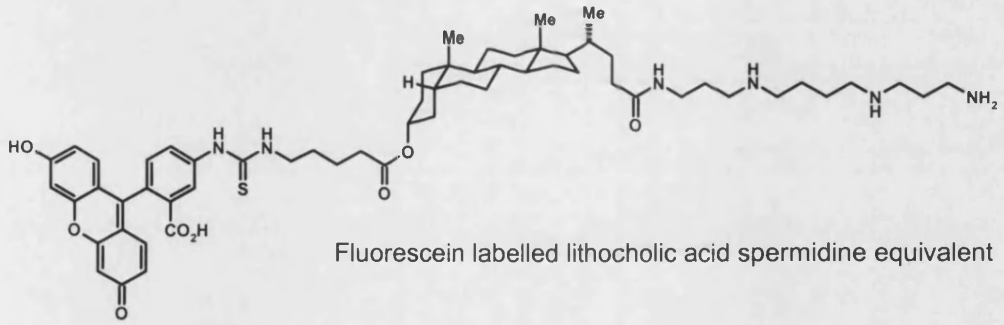


Fmoc-protected aminoester of cholesteryl spermine



Fluorescein labelled lithocholic acid spermidine equivalent

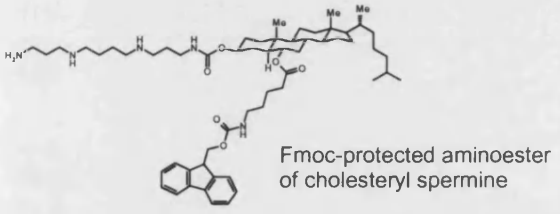
Tri-Boc-protected spermine was acylated with the two chosen steroids in order to afford the 3-cholesteryl carbamate and the lithocholic acid amide of spermine. The introduction of the fluorescent label on cholesteryl carbamate requires hydroboration of the cholesterol Δ^5-alkene functional group. *syn*-Hydroboration (BH_3.dms, oxidised with basic H_2O_2) yielded the 6α-hydroxy steroid, a *trans*-A-B ring junction, displaying H6ax (β) with two *J*ax-ax and one *J*ax-eq at δ = 3.35 ppm, (dt, J = 4.5 and 10.9 Hz). Further elution afforded the minor isomer (~5 %), 6β-hydroxy, a *cis*-A-B ring junction. This 6α-hydroxy and the 3α-hydroxy of the protected lithocholic acid polyamine amide have been esterified with Fmoc-protected 5-aminovaleric acid (DCC/DMAP). Facile removal of the Fmoc-protecting group with TBAF allowed introduction of the fluorophore fluorescein isothiocyanate through the formation of a thiourea. Poly-Boc deprotection (TFA/CH_2Cl_2) and purification (RP-HPLC ODS 60:40 0.1% aq. TFA-MeCN, λ = 495 nm) afforded the target polyamine conjugates. We acknowledge financial support from the EPSRC (studentship to A.P.N.).

Design and Synthesis of Fluorescent Cholesterol and Lithocholic Acid Polyamine Conjugates

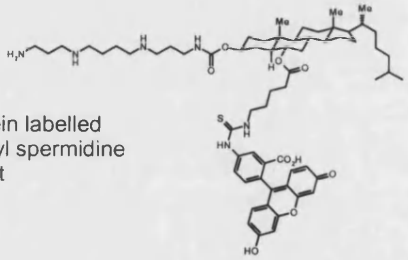
ADRIAN P. NEAL and IAN S. BLAGBROUGH

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

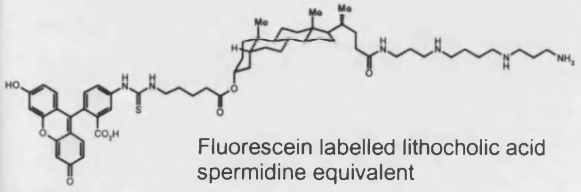
Lipopolyamines are lead compounds for efficient DNA delivery vectors in non-viral gene therapy (Blagbrough et al 1997). 3-Cholesteryl polyamine carbamates (Geall et al 2000) and lithocholic acid polyamine amides (Geall et al 1998) condense DNA, therefore we have designed fluorescent polyamine probes incorporating these steroid moieties. The synthesis of these novel fluorescent lipopolyamines, with both *trans*- and *cis*-AB steroid moieties, is described.



Fmoc-protected aminoester of cholesteryl spermine



Fluorescein labelled cholesteryl spermidine equivalent



Fluorescein labelled lithocholic acid spermidine equivalent

Figure 1. Target fluorescent steroidal lipopolyamines

Tri-Boc-protected spermine was acylated with the two chosen steroids in order to afford the 3-cholesteryl carbamate and the lithocholic acid amide of spermine. The introduction of the fluorescent label on cholesteryl carbamate requires hydroboration of the cholesterol Δ^5-alkene functional group (Neal & Blagbrough 2000). *syn*-Hydroboration ($BH_3.Me_2S$, oxidised with basic H_2O_2) (Tavares et al 1993) yielded the 6α-hydroxy steroid, a *trans*-AB ring junction, displaying H6ax (β) with two *J*ax-ax and one *J*ax-eq at δ = 3.35 ppm, (dt, J = 4.5 and 10.9 Hz). Further elution afforded the minor isomer (~5 %), 6β-hydroxy, a *cis*-AB ring junction (Jung & Johnson 2001). This 6α-hydroxy and the 3α-hydroxy of the protected lithocholic acid polyamine amide have been esterified with Fmoc-protected 5-aminovaleric acid (DCC/DMAP). Facile removal of the Fmoc-protecting group with TBAF (Ueki & Amemiya 1987) allowed the introduction of fluorescein as a fluorophore and conjugation through a thiourea functional group. Poly-Boc deprotection (TFA/CH_2Cl_2) and purification (RP-HPLC ODS 60:40 0.1% aq. TFA-MeCN, λ = 495 nm) afforded the target polyamine conjugates as orange powders.

We thank the EPSRC for a studentship to A.P.N.

Blagbrough, I. S., Carrington, S., Geall, A. J. (1997) Pharm. Sci. 3: 223-233

Geall, A. J., Taylor, R. J., Earll, M. E., Eaton, M. A. W., Blagbrough, I. S. (2000) Bioconjugate Chem. 11: 314-326

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Tavares, R., Randoux, T., Braekman, J.-C., Daloze, D. (1993) Tetrahedron 49: 5079-5090

Jung, M. E., Johnson, T. (2001) Tetrahedron 57: 1449-1481

Ueki, M., Amemiya, M. (1987) Tetrahedron Lett. 28: 6617-6620

Fluorescent Steroidal Lipopolyamine Conjugates For Monitoring Gene Delivery

ADRIAN P. NEAL and IAN S. BLAGBROUGH

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

In non-viral gene therapy, the mechanisms of the intracellular steps are currently poorly understood. Transfection efficiency cannot be improved without a greater knowledge of the molecular detail of intracellular events (Godbey et al 1999a and 1999b). As fluorescent microscopy is potentially useful for the study of lipopolyamine-mediated transfection, the design of fluorescent probes is required. We are studying steroidal polyamine conjugates, cholesteryl carbamates and lithocholic acid amides, as efficient DNA condensing agents that have been designed in order that a variety of fluorophores may be introduced allowing rapid synthesis of a library of fluorescent lipopolyamines (Neal and Blagbrough 2000).

Spectroscopic assays exist for monitoring DNA condensation using such designed probes. These include a fluorescence quenching ethidium bromide assay and a light scattering (λ = 320 nm) assay. Using these techniques, we have shown that our synthetic lipopolyamines condense DNA more efficiently than the natural polyamines spermidine and spermine. A synthetic model lithocholic acid amide of spermine (a spermidine equivalent) derivatised with fluorescein isothiocyanate condensed both calf thymus and synthetic DNA with the same efficiency as spermidine in the ethidium bromide assay. These results help us to design more efficient fluorescent probes for DNA-binding in this area of non-viral gene delivery using alkyl spermine moieties.

Cholesteryl carbamates **1** and **2** and lithocholic acid amides **3** and **4** are spermine equivalents which efficiently condense DNA. Compound **5** is a fluorescent BODIPY derivative of **1**, accessible through hydration of the unsaturated cholesteryl carbamate.

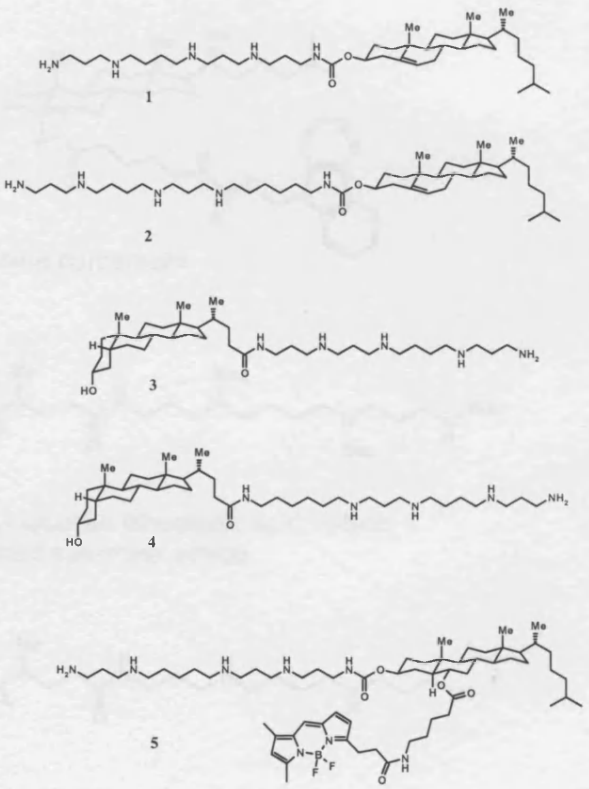

Figure 1. Target fluorescent steroidal lipopolyamine conjugates.

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Godbey W. T., Wu K. K., Mikos A. G. (1999a) Proc. Natl. Acad. Sci. USA, 96: 5177-5181

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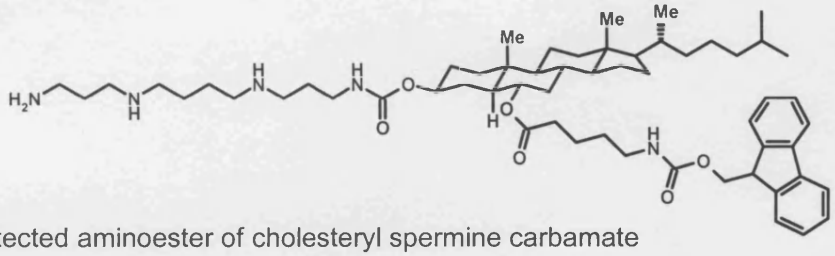
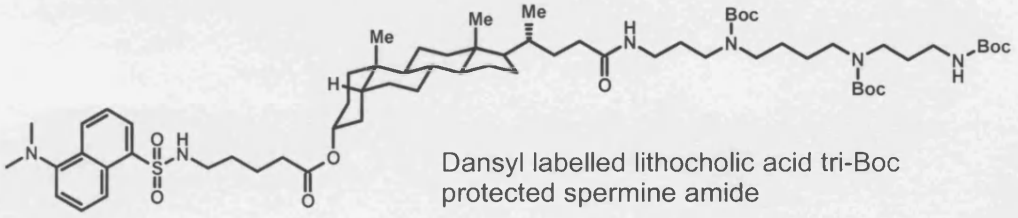
Fluorescent Steroidal Lipopolyamine Conjugates for Monitoring Non-Viral Gene Delivery

Adrian P. Neal and Ian S. Blagbrough
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

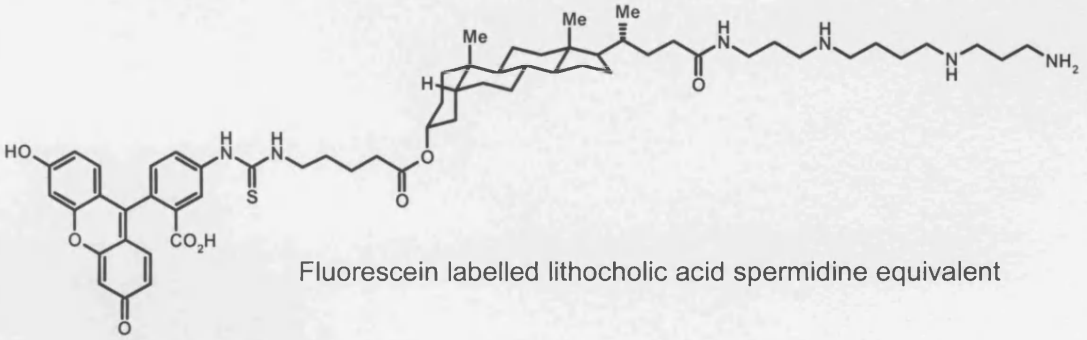
Lipopolyamines are lead vectors designed to achieve efficient DNA delivery in non-viral gene therapy, but the intracellular detail of the processes involved is poorly understood. 3-Cholesteryl polyamine carbamates and lithocholic acid polyamine amides condense polynucleic acids for transfection. In order to provide a rational basis for the design of new efficient vectors, we have designed fluorescent lipopolyamine probes incorporating *trans*- and *cis*-AB steroid moieties. We report the synthesis of these novel analytical tools.



Fmoc-protected aminoester of cholesteryl spermine carbamate



Dansyl labelled lithocholic acid tri-Boc protected spermine amide



Fluorescein labelled lithocholic acid spermidine equivalent

N^1,N^2,N^3-Tri-Boc-protected spermine was acylated with the two chosen steroids in order to afford the 3-cholesteryl carbamate and the lithocholic acid amide. The introduction of a fluorescent label to this 3-cholesteryl carbamate requires hydration of the cholesterol Δ^5-alkene functional group. *syn*-Hydroboration ($BH_3.Me_2S$, oxidised with basic H_2O_2) yielded the 6α-hydroxy steroid, a *trans*-A-B ring junction, displaying H6ax (β) with two *J*ax-ax and one *J*ax-eq at δ = 3.35 ppm, (dt, *J* = 4.5 and 10.9 Hz). Further elution afforded the diastereoisomer (~5 %), 6β-hydroxy, a *cis*-A-B ring junction. This 6α-hydroxy and the 3α-hydroxy of the protected lithocholic acid polyamine amide have been esterified with Fmoc-protected 5-aminovaleric acid (DCC/DMAP). Facile removal of the Fmoc-protecting group (KF/TEA) allowed introduction of fluorophores. Thus, sulfonylation with dansyl (sulfonyl) chloride afforded the lithocholic acid conjugate which will allow monitoring of pH sensitive fluorescence and cellular events during transfection. Fluorescein was introduced as a thiourea to afford cholesteryl and lithocholic acid spermine conjugates. This design and synthesis of a range of fluorescent conjugates allows multicolour confocal imaging of cellular environments and intracellular processes involved in transfection. Poly-Boc deprotection (TFA/CH_2Cl_2 1:9 v/v) and purification (RP-HPLC ODS 60:40 0.1% aq. TFA-MeCN, λ = 495 nm for fluorescein) afforded the target lipopolyamine conjugates. We acknowledge financial support from the EPSRC (studentship to A.P.N.).